CLINICA CHIMICA ACTA

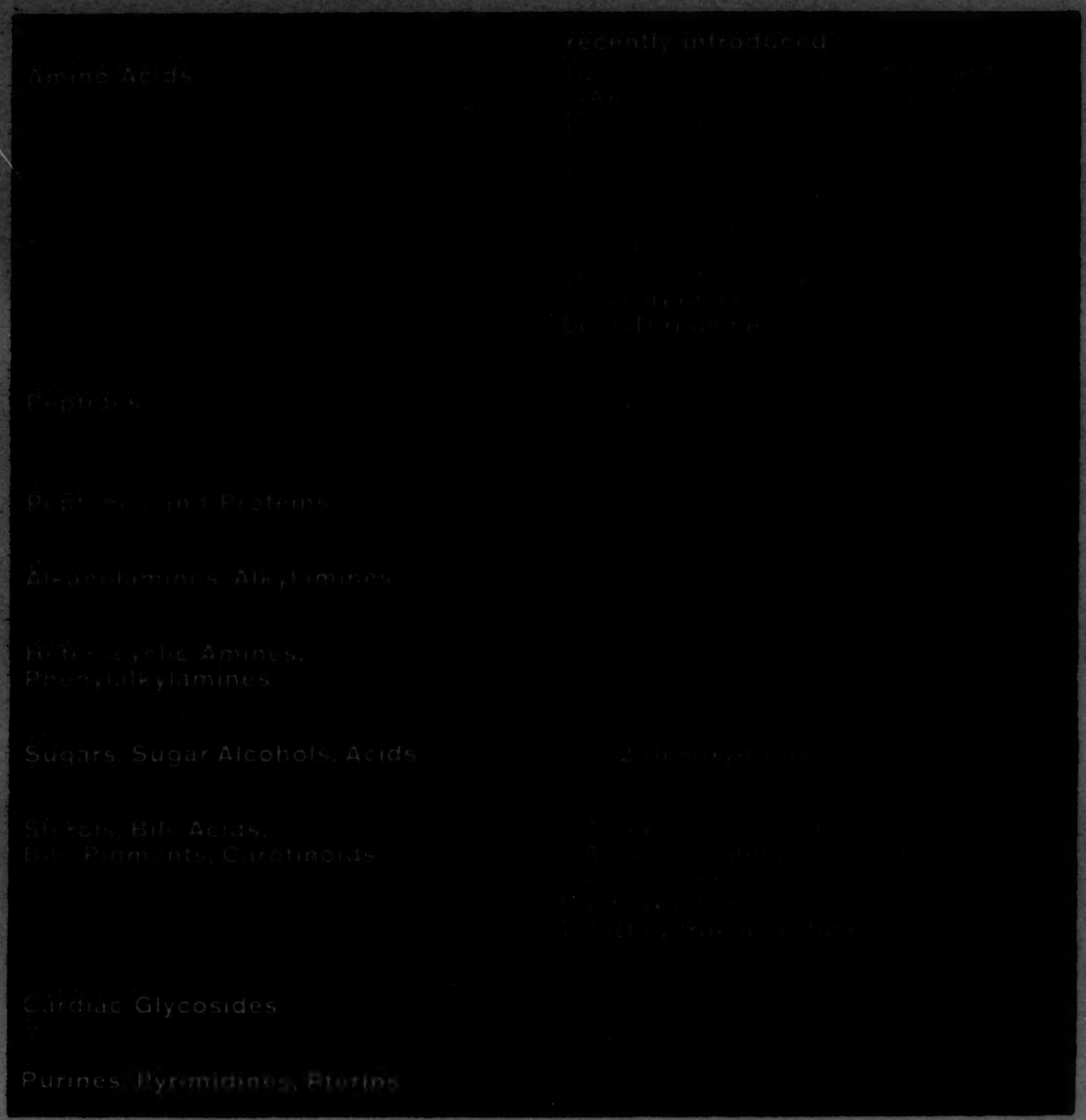
INTERNATIONAL JOURNAL OF CLINICAL CHEMISTRY

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

Clin. Chim. Acta, Vol. 4, No. 3, p. 317-460, May 1959

Biochemica Roche

Over 300 different «Roche» biochemicals are available to meet the growing demands of biochemical research.



Vitamins and Related Substances

The following vitamins are available in pure form: (+)-Biotin; Folic acid; Nicotinic acid; Nicotinamide; Calcium pantothenate; Sodium pantothenate; Vitamin A alcohol cryst.; Vitamin A acetate cryst.; Vitamin A palmitate cryst.; β-Carotene cryst., synth.; Vitamin B_1; Cocarboxylase; Vitamin B_2; Sodium riboflavin-5'-phosphate; Vitamin B_6; Pyridoxamine dihydrochloride; Vitamin B_{12}; Vitamin C; Vitamin D_2; Vitamin D_3; Vitamin E; Vitamin E acetate; Disodium DL-α-toxopheryl phosphate; Vitamin K_1.

Hematology, Blood Coagulation
Plant Growth Regulators
Carcinogenic Substances
Chemicals and Reagents
for Biological and Clinical Research

The catalogue of biochemicals, complete with price-list, may be ordered from
Biochemicals Department
F. Hoffmann-La Roche Ltd. Co., Basle

50045

JUST PUBLISHED

Chromatographic Reviews

PROGRESS IN CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

VOLUME I

Edited by
MICHAEL LEDERER

Chromatography, electrophoresis and the related methods and techniques have undergone enormous expansion in the last years and their applications to the various problems are so numerous that it is hardly possible to obtain an up-to-date survey of the whole field. The editor has therefore taken up the idea of each year inviting various specialists to write a review on those aspects of the subject where such a survey is most needed. These reviews, which have previously appeared in the Journal of Chromatography, will be collected annually, translated into English when originally in French or German, and published in this series. They will thus serve to keep the reader informed of the latest developments in chromatography.

6 1/2 × 9 1/2" xii + 276 pages 114 tables 64 illus. 1959

CONTENTS:

ELSEVIER PUBLISHING COMPANY

110-112 SPUISTRAAT — AMSTERDAM-C

AN IMPROVED TECHNIQUE OF AGAR-GEL ELECTROPHORESIS ON MICROSCOPE SLIDES

R. J. WIEME

Laboratory of the Medical Clinic, University of Ghent (Belgium)

INTRODUCTION

Numerous modifications of the basic electrophoretic techniques of GORDON[1] AND GRABAR[2] using agar-gel as anticonvection medium, have been published in recent years[3-9] (see also [10]). They agree on certain advantages proper to this medium: the absence of adsorption relative to the vast majority of proteins and favorable optical properties[11].

For some time, we have been applying a modified version of the GRABAR technique, employing microscope slides as a support to the gel. The first results, showing its possibilities as an ultramicrotechnique, were demonstrated two years ago[12] and described in some detail elsewhere[13].

We have now succeeded in improving the resolving power of this technique to a degree as to separate with good constancy normal human serum in at least 9 fractions: one (or two) pre-albumin, albumin, α_1-globulin, two α_2-globulins, three β-globulins and a continuous spectrum of γ-globulins. These results present some similarities with the fractionations described by RESSLER who used fluid film electrophoreses (liquid buffer stabilised with 0.13% agar[14, 15]), but the curves we obtain by direct photometry of the stained plates, are much smoother and more regular than the very irregular diagrams published by that author[16].

Pathological human serum may be resolved into even more fractions, especially in the zone of the γ-globulins. The method seems also promising for the analysis of animal serum and tissue proteins. RABAEY AND VERRIEST[17] have shown that *relative* mobilities can be measured with great accuracy under the conditions specified further.

The theoretical considerations that guided us when developing this method are briefly exposed. The technique is described in detail. Finally a few examples are given illustrating the high resolving power of the method.

SOME THEORETICAL CONSIDERATIONS*

(*a*) To separate by electrophoresis two protein fractions of nearly equal mobility, they must be run over a long distance in a short time; small differences in mobility would be revealed over the long path, and the blurring effect of diffusion could not develop in that short time. High voltage gradients seem to be necessary, but they render an efficient cooling system indispensable if a sufficient buffer capacity is to be maintained in the electrophoretic medium. Also should the initial width of the zone containing the protein mixture be kept as small as possible.

(*b*) To maintain a regular distribution of the electric field, losses of water by

* See also ref. [18].

References p. 321

evaporation must be prevented. The electrophoretic plate must be provided with airtight packing.

(*c*) Agar-gel induces an intense electro-endosmotic water transport to the cathode. At the junctions with the electrode vessels containing free buffer, a discontinuity exists that tends to disturb the homogeneity of the salt concentrations[19] and interferes with the homogeneity of the electric field.

To meet the above conditions, we applied a high voltage gradient (of the order of 15 V/cm) on a plate completely immersed in petroleum ether and established direct contact with large agar blocks that bridged the electrophoretic plate with the buffer in the electrode vessels. The mixture to be analysed was applied along a small slit ($\pm$ 1/4 mm) cut in the agar-gel.

Petroleum ether as a cooling medium presents advantages that render it superior to other cooling liquids: it is easy-flowing (η = 0.28 cPs. at 20°), highly volatile (b.p. 25° to 70°) and reasonably inert (solubility in water is much lower than that of toluol). It not only cools by convection but may also, if evaporated, actually refrigerate the electrophoretic plate. In fact, by activating this evaporation with a fan, RABAEY[20] was able to maintain a temperature of + 4° during the whole electrophoretic run.

The electrophoretic plates are layered, face down, on the agar blocks. This ensures a good electric contact and gives a constant distance between the free ends of the electrophoretic track.

ELECTROPHORETIC TECHNIQUE

The following description is primarily concerned with the analysis of human serum proteins. For other materials (tissue proteins, animal sera) some modifications may give better results; especially other buffer compositions should be tried.

Buffer stock solution

pH 8.4, μ = 0.1, sodium veronalate 8.5 g, hydrochloric acid (*N*) 11.5 ml, aq. dist. ad 500 ml. Before use, dilute with distilled water to ionic strength 0.05.

Preparation of the agar plates

Heat the buffer solution in a boiling water bath. Add the agar powder (Difco Special Agar-Noble, 0.80 g for 100 ml buffer) and heat the solution further until entirely clear ($\pm$ 20 min. Pour a portion of the warm agar solution in a petri dish so as to cover the bottom entirely, allow it to cool until a gel is formed (at $\pm$ 40°). When the petri dish is not moved, a perfectly horizontal surface will be obtained. Place the microscope slides (26 × 76 mm) carefully on this surface and pour a new layer of agar in the dish. The thickness of the layer covering the slides, can be controlled by pouring in known volumes to give the required thickness (2 mm). Air bubbles, when present, should be pushed away from the surface of the slides; they dissolve on further cooling. When a solid gel has formed, cover the dish and store in a cool place for at least one day: in the first hours, modifications occur in the agar-gel that influence the electroendosmotic flow markedly.

Application of the sample

Cut out a slide covered with the agar layer, and make one or more small linear grooves into the agar plate by incising with a small gillette blade over 5 mm length,

References p. 321

down to the glass slide. Carefully insert a rectangular piece of rigid filterpaper with a forceps into the slit, so as not to touch the bottom of the groove. After removing the paper, the groove will be dry and the protein solution may be introduced with the aid of a capillary pipette. Calibration, if desired, may be made by weighing the volume of water contained between two marks.

For a 5-mm groove, 1 to 3 μl of the solution is sufficient; for smaller amounts, smaller grooves should be made; quantities of 1/10 to 1/50 μl should preferably be directly deposited *on* the agar layer. For very small tissue fragments ($\pm$ 1 mg fresh weight) homogenisation and extraction can be avoided by introducing them directly in the groove: good separations are obtained, especially at the cathode side of the line of application.

Electrophoretic run

This should be made as soon as possible after the application of the sample. Place the slide with the agar layer facing downward, as a bridge between the agar blocks in the box shown in Fig. 1. The composition of these blocks should be identical to that of the agar-gel used for electrophoresis.

Fill the buffer vessels with the buffer stock-solution and the central tank with petroleum ether (p.a., b.p. 25–70°) and place the cover on the box.

The voltage is applied from a stabilized power supply of conventional design and its value adjusted so as to pass 25 to 35 mA, for which 130 to 150 V at the electrodes are necessary. The voltage must be chosen so as to obtain a rapid separation without disturbing temperature gradients in the agar layer, thus interfering with the pattern of the protein bands.

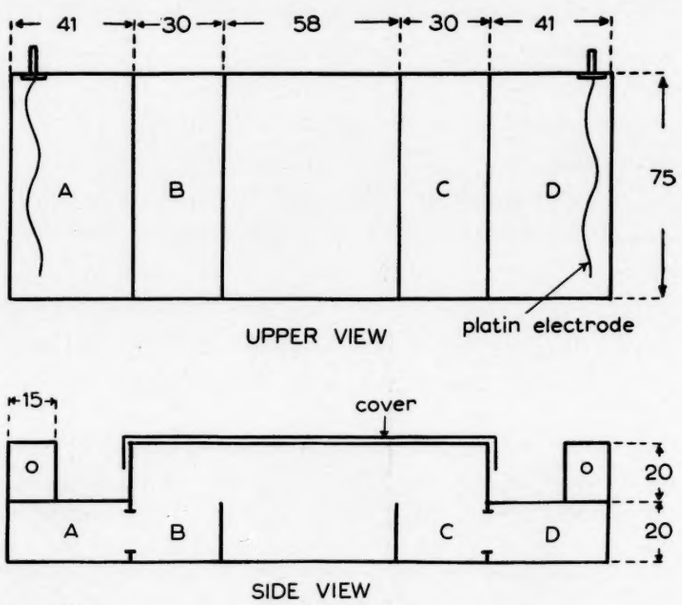


Fig. 1. Scheme of the electrophoresis tank. All dimensions are given in mm. Material: plexiglas (lucite). Tanks A and D are filled with liquid buffer, tanks B and C with buffered agar-gel. Between A/B and C/D large holes ensure good electric continuity. The central tank is filled with petroleum ether. The agar plate is placed, with the agar layer down, as a bridge between B and C.

Electrophoresis proceeds under permanent control of voltage and intensity. The intensity of the electrical current is a sensitive indicator of temperature changes in the agar plate. A rise in this current (the voltage remaining constant) indicates a temperature rise in the plate. A 10% rise can be allowed; if higher, the cover must be removed and some of the petroleum ether evaporated: this immediately cools the plate

References p. 321

down. Generally speaking, such a measure should only be necessary when very steep voltage gradients or low temperatures seem indicated.

Good fractionations are achieved in 25 min. After each electrophoretic run the polarity at the electrodes is reversed. Under this condition, the electrophoresis box with the agar blocks can be used many times (for $\pm$ 1 week).

Fixation

The agar plate must be fixed for 30 min in acetic acid (5%) in 70 vol. % aethanol. Fix as soon as possible after completion of the run.

Drying

Cover the agar plate with a sheet of filterpaper and dry at 37°. Salts and water are in this way completely eliminated. The agar film must be entirely dry and transparent before staining is undertaken.

Staining

Staining in the following solution takes 1 h. Mix in the order given: amidoblack (Bayer) 0.5 g, mercurichloride 5.0 g, acetic acid 5.0 ml, distilled water ad 100.0 ml. Filter before use.

Differentiation

Rinse the plate with 5% acetic acid in distilled water, till complete decoloration of the background. Change the rinsing solution several times.

Mounting

Clarify the preparation in toluol and mount in the usual way in Clarite.

Quantitative estimation

A sensitive photometer whose optical slit is reduced to 1/4 mm may be utilised if a transport mechanism giving steps of 1/4 mm is available. We modified the Eppendorf photometer (Netheler, Hamburg) with its attachment for reading paper electropherograms, so as to insert our plates vertically into the path of the undeflected lightbeam.

Best results are obtained by optical magnification (15 ×) of the preparation*. This is inevitable for very small samples. The image is stepwise displaced before a slit of 1 × 0.3 cm, illuminating a sensitive photocell (photomultiplier cell 931 A) whose output is a measure of the light transmitted. Optical densities are plotted on graph paper and the curve is integrated in the usual way. The limits of measurability seems to be 10^{-7} g protein when an amidoblack stain is utilised.

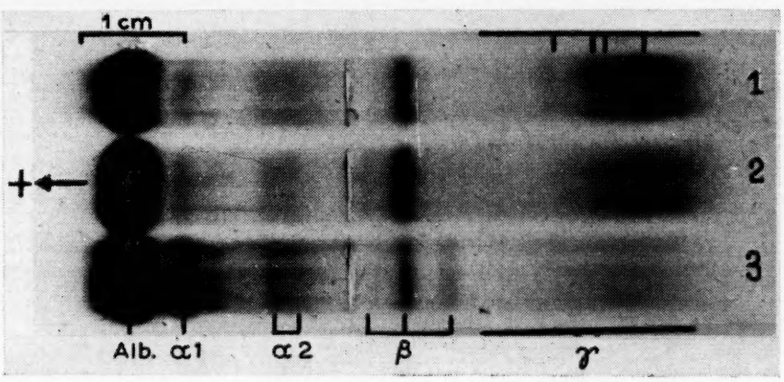


Fig. 2. Pherograms obtained in three pathological sera, on one microscope slide. Diagnosis: (1) chronic infection of the bile ducts, (2) hepatitis, (3) lymphatic leukaemia. The line of application corresponds to the small slits in the agar film. In (1) subfractions are indicated in the γ-globulin range. For further details, see text.

* We used a slightly modified "Lanameter" microscope (Reichert, Vienna).

References p. 321

Very irregular spots cannot be correctly scanned by direct photometry when a fixed slit slength is utilised. This is only possible by scanning the whole spot through a very small slit. A scanning-integrating densitometer may be used[21].

EXAMPLES

Fig. 2 represents three electropherograms obtained with this technique on three different pathological human sera. In each of them, the α_2-globulins are resolved in two components. The separation of albumin and α_1-globulin is good. A fraction between the α_2-globulins and the β_1-globulin can be seen on two of the electropherograms. In 1, four to five subfractions are found in the γ-globulin range. The prealbumins cannot be studied here as they have run too far ahead. Fig. 2 illustrates the great variety one finds during the analysis of pathological human sera and at the same time demonstrates the outstanding reproducibility of the mobilities in the different protein zones.

One drawback must not be overlooked: the intensity of the albumin band is such that the extinction values lie beyond the zone of accurate colorimetry. The relative values obtained for this fraction are too high (70–80% in normal human serum). Though the reason for this is not quite clear, with a nigrosin stain (water-soluble Nigrosin, Geigy) the percentages agree better with accepted values.

SUMMARY

A technique of agar-gel electrophoresis possessing a high resolving power is described whereby a minimum of nine fractions are revealed in normal human serum. This is achieved by using a steep voltage gradient so as to complete the electrophoretic separation in less than 30 min. A very efficient, inexpensive cooling liquid was found in petroleum ether. Water losses due to evaporation are completely avoided. The electro-endosmotic flow is regularized by interposing large agar blocks between the electrophoretic plate and the electrode vessels. The method seems to be especially promising for the study of tissue proteins and of animal serum proteins.

REFERENCES

1 A. H. GORDON, B. KEIL AND K. SEBESTA, *Nature*, 164 (1949) 498.
2 P. GRABAR AND C. A. WILLIAMS, JR., *Biochim. Biophys. Acta*, 10 (1953) 193.
3 A. BUSSARD AND D. PERRIN, *J. Lab. Clin. Med.*, 46 (1955) 689.
4 J. SCHEIDEGGER, *Intern. Arch. Allergy, Appl. Immunol.*, 7 (1955) 103.
5 J. URIEL AND J. SCHEIDEGGER, *Bull. soc. chim. biol.*, 37 (1955) 165.
6 K. V. GIRI, *Naturwissenschaften*, 43 (1956) 36.
7 K. V. GIRI, *J. Indian Inst. Sci.*, 38 (1956) 190.
8 CH. WUNDERLY AND V. BUSTAMANTE, *Klin. Wochschr.*, 35 (1957) 758.
9 F. A. PEZOLD AND H. THOMAS, *Z. ges. exptl. Med.*, 129 (1957) 412.
10 A. J. CROWLE, *J. Lab. Clin. Med.*, 48 (1956) 642.
11 R. J. WIEME, *Behringwerke Mitteilungen*, 34 (1958) 27.
12 R. J. WIEME, *Proceedings of the IVth Symposium on Protides of Biological Fluids*, St. Jans Hospitaal, Brugge, De Tempel, Brugge, 1956, p. 27.
13 R. J. WIEME AND M. RABAEY, *Naturwissenschaften*, 44 (1957) 112.
14 N. RESSLER AND S. D. JACOBSON, *Science*, 122 (1955) 1088.
15 N. RESSLER AND B. ZAK, *Clin. Chim. Acta*, 1 (1956) 392.
16 N. RESSLER AND S. D. JACOBSEN, *Clin. Chim. Acta*, 2 (1957) 372.
17 M. RABAEY AND G. VERRIEST, *Ann. soc. roy. zool. Belg.*, 88 (1958) 373.
18 R. J. WIEME, *Thesis*, University of Ghent, Belgium, in the press.
19 R. D. MILLER, *Science*, 122 (1955) 373.
20 M. RABAEY, personal communication.
21 R. J. WIEME, *J. Chromatog.*, 1 (1958) 166.

Received July 23rd, 1958

THE INTERACTION BETWEEN DYES AND SERUM PROTEINS IN PAPER ELECTROPHORESIS

SOME QUANTITATIVE PROBLEMS

V. SCARDI AND V. BONAVITA

*Institute of Human Physiology, University of Naples (Italy)**

The aim of this paper is to elucidate some problems connected with the quantitative estimation of serum proteins by paper electrophoresis. Although far from being complete, it summarizes almost all the results obtained up to the present in this laboratory, following a line that seems interesting. A complete review of the literature would require following the steps of previous workers in this field, who describe more or less extensively many well-known aspects of the quantitative estimation of serum proteins by paper electrophoresis, *e.g.* KÖIW *et al.*[1], GRASSMANN AND HANNIG[2], JENCKS *et al.*[3], BLOCK *et al.*[4]. Extensive discussions on the subject are to be found in reports of special symposia[5, 6].

Obviously, the quantitative estimation of serum proteins by paper electrophoresis requires a stoichiometric relationship between the denaturated proteins and the dye. Such a relationship was observed only in very special conditions. Moreover, it is necessary that the dye-binding capacity of all protein fractions be the same, or at least that any difference be constant. In the latter case a correction factor that is valid in very different experimental conditions could counterbalance the difference in the dye-binding capacity of the various protein fractions. Correction factors were proposed by CREMER AND TISELIUS[7], KUNKEL AND TISELIUS[8], KÖIW *et al.*[1], GRASSMANN AND HANNIG[2], who assumed that the various serum proteins have different dye binding capacities, by comparison with free electrophoresis data. HARDWICKE[9], however, found no such difference between serum albumin and γ-globulin.

The disagreement in the findings may be attributed to the diverse experimental conditions employed by the various authors and to the fact that the primary role of the ratio between protein concentration and surface area on paper (surface concentration) was disregarded. HARDWICKE[9] acknowledged the significance of such a parameter in connection with the problem of "packing". A complete analysis of the behaviour of a number of dyes with respect to the two main serum proteins, *i.e.* albumin and γ-globulin, has, however, only been made by us. We followed the experimental scheme that FRAGLEN AND MARTIN[10] described for bromocresol green.

Thus, this paper presents both published and unpublished data, and describes a suitable procedure for the quantitative estimation of serum proteins in paper electrophoresis.

---

* Postal address: Istituto di Fisiologia Umana, Università di Napoli, S. Andrea delle Dame 8, Napoli, Italia.

References p. 328

EXPERIMENTAL

Serum proteins

Human albumin and γ-globulin were a gift of Istituto Sieroterapico Italiano (Naples). The homogeneity of both fractions was checked by free electrophoresis according to TISELIUS. The protein concentration was determined by the biuret method[11], using a solution of human serum albumin (cryst.) as standard.

Human serum

A pool of human sera from 30 blood donors was used in our experiments.

Dyes

The dyes used in our experiments were the following: Azocarmine B (Azo) (Light), Bromophenol Blue (BPB) (Merck), Amidoschwarz 10 B (AS) (Bayer).

Paper

The paper was Whatman No. 1 for chromatography.

Estimation of dye uptake

Dilutions ranging from 0.5 to 5% were prepared from the γ-globulin and the albumin stock solutions in 0.15 *M* NaCl. By means of an Agla microsyringe, each solution was applied, in duplicate, to paper strips 4 cm wide, as spots of 5, 10, 20, 30 and 40 μl. The paper strips were divided by pencil into squares of 4 × 4 cm, and the protein solutions were applied onto the center of each square. The protein spots were first dried in air, then at 105° in the oven for 15 min. Finally, they were placed in the staining baths. The staining procedures were the following:

(*a*) *Azocarmine B.* According to the procedure described by SCARDI *et al.*[12].

(*b*) *Amidoschwarz 10 B.* According to the procedure described by GRASSMANN AND HANNIG[2].

(*c*) *Bromophenol blue.* The staining bath was prepared as follows: 0.1 g of BPB was dissolved in 15 ml 95% ethanol and diluted by dropwise addition of 30 ml distilled water (sol. 1). 5 g of $ZnSO_4 \cdot 7H_2O$ were dissolved in 50 ml dist. water (sol. 2). These two solutions were gradually mixed and then 5 ml of glacial acetic acid were added. Staining in this bath takes at least 30 min. The rinsing bath was a 5% solution of acetic acid. It is necessary to rinse at least three times[13].

After staining, rinsing and drying, the long and the short axis of each spot were measured, and the surface area was calculated with the formula: $S = D \times d \times \pi/4$. The paper strips were cut in square pieces 4 × 4 cm and the dye eluted. Azo and BPB were eluted in 0.01 *N* NaOH for 30 min, while AS was eluted in 0.1 *N* NaOH for 60 min. The amounts of eluted dyes were determined spectrophotometrically at different wavelengths (Azo, 540 mμ; BPB, 595 mμ; AS, 625 mμ). The corresponding values were compared with standard curves for each dye.

Calculation of results

For each protein concentration two diagrams were made. In one the weight of the eluted dye was plotted against the protein weight, in the other against the area of each spot. The best fitting curves were drawn and the slopes measured; these represent in the one case the weight of dye bound per unit weight of protein, and in the other the weight of dye distributed over the unit area of the protein spot.

References p. 328

Paper electrophoresis of the serum

This was carried out on 4-cm wide paper strips in horizontal-strip type cell[14]. Barbital buffer, pH 8.6, ionic strength 0.06, was used. Separation was carried out with a constant current of 1.5 mA per strip, at room temperature for 3.5–4 h. The strips were then removed from the migration cell and dried at 105° for 15 min before staining according to the procedures mentioned above.

Scanning

The stained strips were scanned with an EEL apparatus (Evans Electroselenium Ltd.), without "clearing", light filters being used for each dye. The instrument was equipped with a rule recalibrated according to SMITH[15]. Quantitative evaluation of the scanning patterns was carried out by cutting and weighing.

Elution

After scanning, the strips were cut into the desired segments and eluted as previously described for each dye.

Quantitative evaluation of electrophoretic patterns

The albumin content of the sample was calculated by subtracting the globulin content from the total protein content, both determined by the biuret method[11]. The globulins were determined after precipitation by 27% saturation with sodium sulphate at 37° for 3 h. On the other hand, the amounts of the different globulins, expressed as percentage of the total globulins, were calculated from elution or scanning data. From these values it was possible to find the absolute value of each fraction by reference to the total globulin content, previously determined by the biuret method. A simple calculation enables us to report the values of the globulin fractions as percentages of the total protein content of the sample.

Free electrophoresis

This was carried out with a Perkin-Elmer apparatus model 38, using a barbital buffer, pH 8.6, ionic strenght 0.1. The current intensity was 10 mA, the migration time 2 h.

RESULTS

Dye uptake

Figs. 1 and 2 show Azo uptake by albumin and γ-globulin in terms of μg Azo/μg protein and μg Azo/mm^2 spot, respectively, as a function of protein concentration. The curves in Fig. 1 are not superimposed but run fairly parallel. The dye uptake by γ-globulin appears to be less than that of albumin at any concentration. Figs. 3 and 4 show similar plots for AS, and Figs. 5 and 6 for BPB. Also for AS and BPB the uptake by γ-globulin is less than that by albumin, but the curves in Figs. 3 and 5 do not run as closely parallel as in Fig. 1.

Reliability of the quantitative evaluation of electrophoretic patterns

Three methods were used to test the reliability of the results obtained by the proposed procedure.

1. *Comparison with usual procedures.* Table I shows the percentages of different protein fractions, according to the method used for each dye in the routine electro-

References p. 328

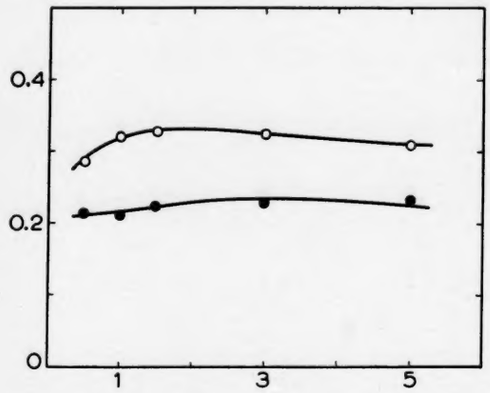


Fig. 1. Uptake of Azo by albumin (–o—o–) and by γ-globulin (–●—●–) per unit weight. Abscissa: protein concentration, g/100 ml. Ordinate: μg Azo/μg protein.

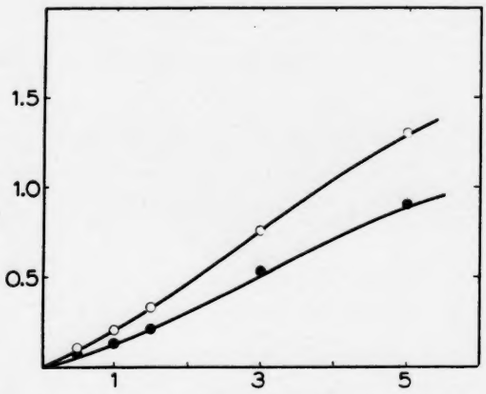


Fig. 2. Uptake of Azo by albumin (–o—o–) and by γ-globulin (–●—●–) per unit area. Abscissa: protein concentration, g/100 ml. Ordinate: μg Azo/mm^2 protein spot.

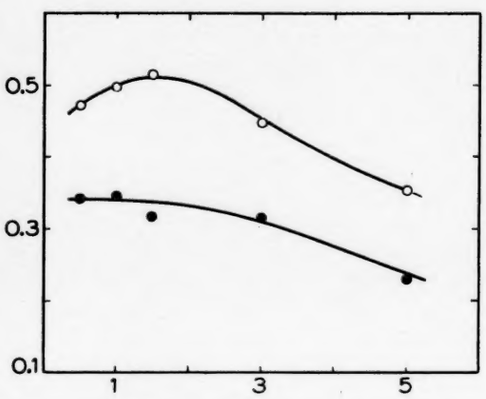


Fig. 3. Uptake of AS by albumin (–o—o–) and by γ-globulin (–●—●–) per unit weight. Abscissa: protein concentration, g/100 ml. Ordinate: μg AS/μg protein.

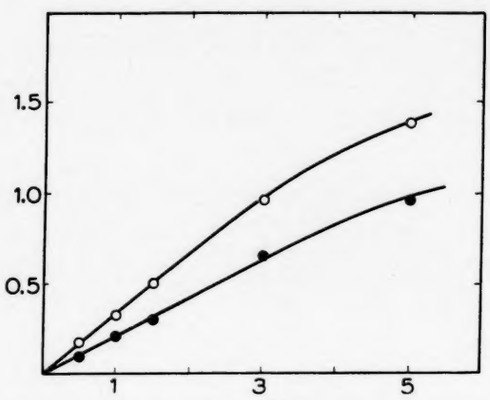


Fig. 4. Uptake of AS by albumin (o—o–) and by γ-globulin (–●—●–) per unit area. Abscissa: protein concentration, g/100 ml. Ordinate: μg AS/mm^2 protein spot.

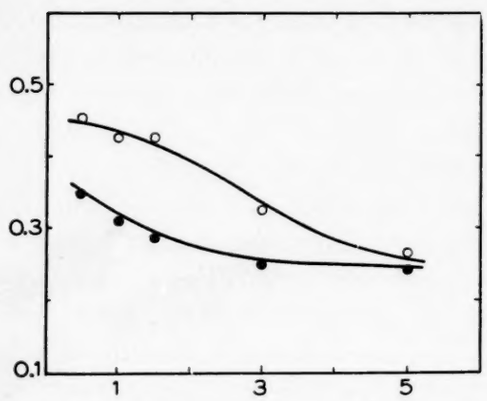


Fig. 5. Uptake of BPB by albumin (–o—o–) and by γ-globulin (–●—●–) per unit weight. Abscissa: protein concentration, g/100 ml. Ordinate: μg BPB/μg protein.

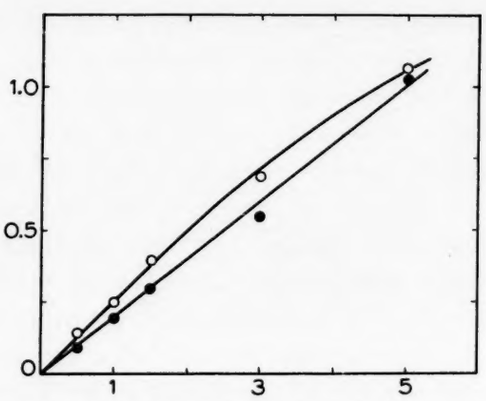


Fig. 6. Uptake of BPB by albumin (–o—o–) and by γ-globulin (–●—●–) per unit area. Abscissa: protein concentration, g/100 ml. Ordinate: μg BPB/mm^2 protein spot.

References p. 328

TABLE I

COMPARISON OF ELUTION AND SCANNING DATA OBTAINED WITH THE ROUTINE PROCEDURE

Fraction	*Elution*			*Scanning*		
	Azo	*Dye AS*	*BPB*	*Azo*	*Dye AS*	*BPB*
Albumin	47.3	44.1	56.3	40.1	43.7	53.6
α_1-globulin	7.4	8.5	6.0	7.0	5.8	5.1
α_2-globulin	8.1	9.6	7.5	9.3	9.6	7.1
β-globulin	14.5	13.7	11.8	16.2	15.5	14.5
γ-globulin	22.7	24.1	18.4	27.4	25.4	19.7

The values represent the averages of at least 4 electrophoretic patterns obtained simultaneously.

TABLE II

COMPARISON OF ELUTION AND SCANNING DATA OBTAINED WITH THE PROPOSED PROCEDURE

Fraction	*Elution*			*Scanning*		
	Azo	*Dye AS*	*BPB*	*Azo*	*Dye AS*	*BPB*
Albumin	55.0	55.0	55.0	55.0	55.0	55.0
α_1-globulin	6.5	6.6	6.6	5.7	5.0	6.0
α_2-globulin	7.0	7.4	7.5	6.5	6.1	6.7
β-globulin	11.2	10.8	12.0	11.9	12.1	13.4
γ-globulin	20.5	20.2	19.1	20.9	21.9	18.9

The values represent the averages of at least 4 electrophoretic patterns obtained simultaneously.

phoretic analysis of the serum. Table II shows the percentages of albumin and globulin fractions, obtained with the procedure proposed by us. The albumin value was calculated by taking the difference between the total protein content and the globulin content of the sample, both determined colorimetrically. No correction was made in either procedure to account for background and albumin tailing. Assuming that the albumin tail was homogeneous, an attempt was made to correct the per cent values of the different fractions. After the correction, the albumin value is increased, but there is no agreement with the A/G ratios determined colorimetrically. On the other hand it will be seen in Table II that the per cent values are in agreement.

2. *"Decapitation" of serum.* A sample of the pooled sera used in our experiments was "decapitated" by salt precipitation of the globulins, while the albumin fraction was subsequently precipitated with trichloroacetic acid and redissolved in barbital buffer. The globulin precipitation appeared to be complete, as confirmed by free electrophoresis. Owing to the aggregation of the α_2- and β-fractions, it was, however, not possible to calculate the per cent values of the globulin fractions in this experiment.

3. *Dilution of the serum.* On diluting the serum up to 1:4 no variation of per cent values was observed, provided the protein amount on the paper was kept constant by proportionally increasing the sample volume, starting from 5 μl for undiluted serum.

References p. 228

DISCUSSION

A complete analysis of experimental data concerning the interaction between the two main serum proteins and different dyes shows that the stoichiometry of the reaction is modified by many factors and, mainly, by the surface concentration of proteins. On varying the above mentioned parameter, the uptake by proteins of AS, Azo and BPB in aqueous solution seems to be fairly constant. Similarly, the difference in the dye-binding capacity of serum albumin and γ-globulin was found to be fairly constant, also in very different experimental conditions. Other dyes, previously examined by us (BPB in ethanol[12], Light Green[16] and Nigrosine[17]) did not behave like this. It seemed therefore convenient to stain pooled sera with AS, Azo and BPB after electrophoretic migration. We obtained confirmation of the difference in dye-binding capacity of globulins and albumin. If this difference were constant, it would be possible to use a correction factor for each dye; the difference is, however, not really constant, except for Azo. The correction factor mentioned here is not the same as that proposed by many authors, based on reference values obtained by free electrophoresis (see above), but it is derived from direct determination of the difference in the dye-binding capacity of albumin and γ-globulin.

Since albumin is the main serum protein, it is evident that errors in its calculation due to 'packing' and 'tailing', may greatly alter the per cent values of the globulin fractions. For this reason, it seemed desirable to limit the calculation of the pattern to the values of the globulin fractions expressed as percentages of the total globulins (see above). Though the proposed procedure is open to criticism, the number of the errors is markedly reduced. In the case of dyes that give a background on paper (AS and BPB), the background does not modify the per cent value of the various globulin fraction to the same extent, owing to the different surface areas of the globulin fractions on paper. The longer the run of proteins, the higher the error. Generally speaking it must be noted that correction for the background is not practically possible. Where protein is adsorbed on paper, the latter cannot adsorb the dye. The most that can be done is to subtract the background of a corresponding area of paper on which no protein has been adsorbed. Such subtraction is possible for geometric figures but not for irregular areas like those observed on paper strips after electrophoretic migration. In scanning, as is known, the background is automatically subtracted, and this constitutes a source of error.

Similar considerations are possible for albumin tailing. As the tail is independent of the albumin concentration, simple serum dilution eliminates the 'packing' but increases the role of 'tailing' as a source of error. Since the albumin tail represents only 5%[3] of the total albumin, it is clear that the error caused by neglecting the relative correction is very small. It is probable that such an error is not greater than that made by empirical evaluation of the tail. As it gives no background, Azo seems to be the best dye for the procedure proposed by us. The main source of error will be the albumin tail.

The empiricism, however, in cutting the paper strips for the elution and the scan patterns for measuring areas appears to present another unavoidable source of error.

ACKNOWLEDGEMENT

Thanks are due to Professor M. FIORENTINO for stimulating discussions.

References p. 328

SUMMARY

The great influence of the surface concentration of proteins on paper on the dye uptake was studied for a number of protein stains (Azocarmine B, Amidoschwarz 10 B, Bromophenol Blue, Light Green, Nigrosine). On the basis of the results, a simple procedure is described for the quantitative evaluation of electrophoretic patterns.

REFERENCES

1 E. KÖIW, G. WALLENIUS AND A. GRONVALL, *Scand. J. Clin. & Lab. Invest.*, 4 (1952) 47.
2 W. GRASSMANN AND K. HANNIG, *Z. physiol. Chem., Hoppe–Seyler's*, 290 (1952) 1.
3 W. P. JENCKS, M. R. JETTON AND E. L. DURRUM. *Biochem. J.*, 60, (1955) 205.
4 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press Inc., New York, 1955.
5 *Ciba Foundation Symposium on Paper Electrophoresis*, J. & A. Churchill Ltd., London, 1956.
6 *Atti del 1° Simposio sulla elettroforesi su carta*, Suppl. N° 1 alla *Rass. med. sper.*, 3 (1956).
7 H. D. CREMER AND A. TISELIUS, *Biochem. Z.*, 320 (1950) 273.
8 H. G. KUNKEL AND A. TISELIUS, *J. Gen. Physiol.*, 35 (1951) 89.
9 J. HARDWICKE, *Biochem. J.*, 57 (1954) 166.
10 G. T. FRAGLEN AND N. H. MARTIN, *Biochem. J.* 57 (1954) 627.
11 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
12 V. SCARDI, A. ORENGO AND E. ROMANO, *Boll. soc. ital. biol. sper.*, 31 (1955) 1327.
13 M. FIORENTINO, personal communication.
14 V. SCARDI, *Boll. soc. ital. biol. sper.*, 30 (1954) 533.
15 R. L. SMITH, *Clin. Chim. Acta*, 1 (1956) 122.
16 V. BONAVITA AND V. SCARDI, *J. Chromatog.*, 1 (1958) 285.
17 V. SCARDI AND V. BONAVITA, *J. Chromatog.*, 1 (1958) 287.

Received July 5th, 1958

THE COLORIMETRIC ESTIMATION OF SERUM FATTY ESTERS

HAROLD L. ROSENTHAL*, MARI LOU PFLUKE AND JOSEPH CALLERAMI
Division of Biochemistry, Department of Pathology
Rochester General Hospital, Rochester, N.Y. (U.S.A.)

The method of STERN AND SHAPIRO[1] makes it possible to determine the fatty acid ester content of blood serum quickly and accurately. The method is based on the alkaline hydroxylaminolysis of carboxylic acid esters[2-4] and other ester linkages[5, 6] to form hydroxamic acids. The colorless hydroxamic acids form highly colored complexes with ferric ion in acid solution that are suitable for photometric estimation. Although the reaction is simple, rapid, and well suited for use in clinical laboratories, the method suffers from the lack of a suitable standard with which to make photometric comparisons. In all reports to date, olive oil or triolein is standardized by saponification, and the oil is then used as the primary standard. Since the triolein slowly undergoes oxidative hydrolysis, the oil requires frequent restandardization, a time consuming procedure.

A suitable crystalline standard, readily obtainable and stable for long periods of time would enable the average clinical laboratory to routinely perform fatty ester determinations. Since more than 50% of the serum esters is in the form of esterified cholesterol, it occurred to us that cholesteryl acetate would serve as a satisfactory standard for this determination. A study of the procedure using cholesteryl acetate as the standard was undertaken and forms the basis for this report.

MATERIALS AND METHODS

Reagents

Cholesteryl acetate standard.

Stock standard: Dissolve 215 mg dry cholesteryl acetate (Eastman Kodak Co.) in Bloor's solvent and dilute to 200 ml with Bloor's solvent. (1 ml = 2.5 μmoles ester.)

Working standard: Dilute 20 ml of stock standard to 100 ml with Bloor's reagent. (1 ml = 0.5 μmole.)

Bloor's solvent. Mix 3 parts ethyl alcohol (95%) and 1 part ethyl ether.

Hydroxylamine solution (2 M). Dissolve 14.4 g hydroxylamine hydrochloride (Eastman Kodak Co.) in water and dilute to 100 ml. The acid solution is stable at room temperature for at least 1 month.

Sodium hydroxide solution (3.38 N). Dilute 75 ml saturated sodium hydroxide to 350 ml with water. Titrate against standardized acid and adjust to 3.38 *N*. Store in polyethylene containers.

Hydrochloric acid solution (3.8 N). Dilute 40 ml conc. hydrochloric acid to 90 ml water. Titrate against standardized base and adjust to 3.8 *N*.

---

* Present address: Washington University School of Dentistry, St. Louis, Mo.

References p. 333

Ferric chloride solution. Dissolve 10 g ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in sufficient 0.1 *N* hydrochloric acid to make 100 ml.

EXPERIMENTAL DETAILS

The absorption spectra of the ferric hydroxamate complex formed with cholesteryl acetate or serum extracts (Fig. 1) are similar with maximum absorbance at 520 mμ. On the other hand, the spectrum of the reaction product for olive oil shows a maximum at 500 mμ and the entire absorption spectrum is shifted towards lower

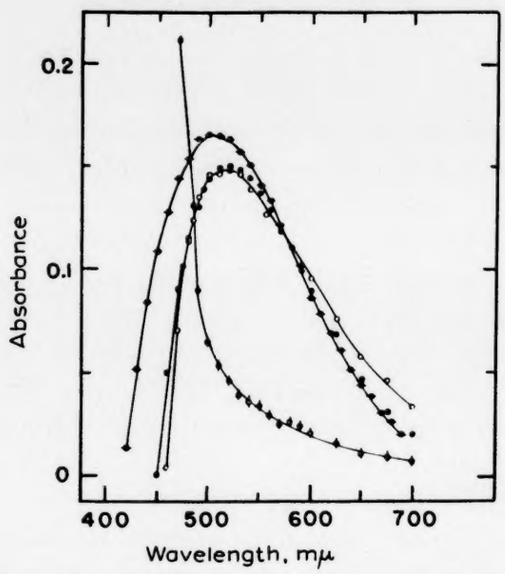


Fig. 1. The absorption spectra of various esters as ferric hydroxamate complex. Reagent blank ϕ; cholesteryl acetate -●-; serum extract O; olive oil ⊖. The spectra were determined with a Beckman DU Spectrophotometer with 1-cm light path.

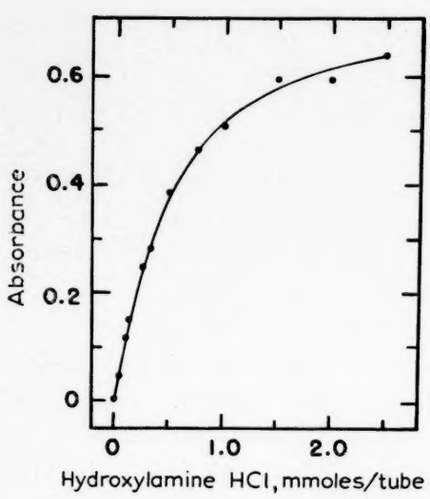


Fig. 2. The effect of hydroxylamine concentration on color formation. Reactions were performed as outlined in the procedure with 5 μmoles of olive oil per tube.

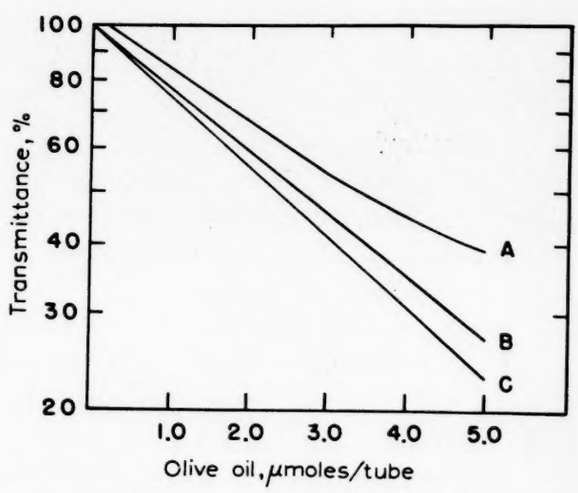


Fig. 3. Relationship between ester concentration and transmittance at various hydroxylamine hydrochloride concentrations. The hydroxylamine hydrochloride concentrations for Curve A – 0.5 mmoles/tube; Curve B – 1.0 mmoles/tube; and Curve C – 1.5 mmoles, 2.0 mmoles and 2.5 mmoles/tube.

References p. 333

wave lengths. The absorbance of the reagent blank decreases sharply from 460 mμ to 700 mμ and becomes relatively small in the region most suitable for photometric measurements. However, the colored complexes gradually appear to change color, on standing at room temperature for 6 to 8 h, due to the reduction of yellow ferric ion to colorless ferrous ion by the excess hydroxylamine in solution. Although the ferric chloride is slowly reduced, the absorptivity of the test solutions remain constant for about 2 h, when determined against the reagent blank.

The concentration of hydroxylamine hydrochloride necessary to produce maximum color formation was determined by adding graded quantities of the reagent to 5 μmoles of olive oil by the proposed procedure. As shown in Fig. 2, color formation increases rapidly with increasing hydroxylamine hydrochloride concentration and reaches completion between 1.5 and 2.5 mmoles/tube.* The effect of hydroxylamine hydrochloride concentration on the linearity of color production (Fig. 3) indicates that excess reagent must be present in order to drive the reaction to completion. At low concentrations, (0.5 mmoles/tube) the formation of color does not obey Beer's law (Curve A) and makes the reaction unsuitable for photometric analysis. Maximum color is obtained with 1.5 mmoles/tube and the incorporation of higher concentrations of hydroxylamine hydrochloride (2.0 and 2.5 mmoles/tube) yield identical results (Curve C). The concentration of hydroxylamine hydrochloride selected by us (2.0 mmoles/tube) compares favorably with that reported by STERN AND SHAPIRO[1] but is higher than that used by TOMPSETT AND TENNANT[2]. Although olive oil was used for most of the preliminary experiments, cholesteryl acetate yielded similar results in succeeding trials.

The alkalinity for the hydroxylation reaction is not critical between pH 8 and pH 12 but is optimal at pH 10. On the other hand, the final acidity for the formation of the ferric hydroxamate colored complex is critical between pH 1 and pH 2. At reactions more acid than pH 1, color formation is depressed and at reactions above pH 2, ferric hydroxide may precipitate and invalidate the analysis.

With this information at hand, the procedure adopted for routine use is as follows:

1. Extraction of fatty acid esters from serum

To about 15 ml of Bloor's solvent in a 25-ml volumetric flask, add 0.5 ml serum with shaking. Bring solution just to boiling on steam bath, cool to room temperature and dilute to volume with Bloor's solvent. Filter extract through fat-free filter paper with the funnel covered by a watch glass to minimize evaporation.

2. Determination of fatty acid esters

Place 5 ml of extract into a 50-ml erlenmeyer flask. A reagent blank (5 ml Bloor's solvent) and working standard (5 ml) are carried through with each determination. Add 1 ml hydroxylamine hydrochloride solution. Mix and add 1 ml sodium hydroxide solution. Mix, cork, and allow to stand for 20 to 30 min at room temperature. Add 1 ml hydrochloric acid solution. Mix and add 1 ml of ferric chloride solution. Mix and

* The term "per tube" indicates amounts of fatty acid or hydroxylamine per 5 ml of "extract" analyzed as described under *Determination of fatty acid esters* and transferred to a photometer cuvette tube. Micromoles of fatty acid esters are expressed in terms of micromoles of the fatty acids.

References p. 333

allow color to develop for 10 min. Determine the absorbance of standard and unknown samples at 520 mμ with the photometer adjusted to 0 absorbance with the reagent blank. The data presented in this report were obtained with a Bausch and Lomb Spectronic 20 instrument, but any photometer transmitting light in this region is satisfactory.

RESULTS

In 5 separate determinations, cholesteryl acetate yielded color values equivalent to 98.1% (S.D. = 2.1) of the color formed by a carefully standardized sample of olive oil. To further confirm the applicability of cholesteryl acetate as a standard, a series of determinations were performed comparing the photometric method with an accepted extraction, saponification and titration procedure[7]. For six pooled serum samples, obtained from the routine laboratory, the photometric method described in this report yielded results equivalent to 98.9% (S.D. = 4.1) of the values obtained by the saponification analysis.

TABLE I

CONSECUTIVE DETERMINATIONS OF A SERUM SAMPLE ANALYZED WITH THE PROPOSED PROCEDURE

Trial	*mmoles/l*	*Trial*	*mmoles/l*
1	13.4	9	13.5
2	13.8	10	13.1
3	13.5	11	13.5
4	13.8	12	13.5
5	12.9	13	13.0
6	13.1	14	14.3
7	13.6	15	12.8
8	13.4		

Mean = 13.4; Range = 12.8 to 14.3; Standard deviation = 0.38; Coefficient of variation = 2.83%.

The reproducibility of the proposed procedure was studied by analysing a pooled serum sample for 15 consecutive determinations. The results, (Table I), indicate a high degree of precision as shown by the coefficient of variation of 2.83%.

Recovery studies, performed by adding known amounts of olive oil or cholesteryl acetate to serum samples, were adequate and ranged from 97% to 102% of the added material as shown in Table II.

TABLE II

RECOVERY OF CHOLESTERYL ACETATE OR OLIVE OIL ADDED TO SERUM AND ANALYZED WITH THE PROPOSED PROCEDURE

Substance	*No. of Detn.*	*Added (mmoles/l)*	*Recovered per cent*
Olive oil	6	2	102 ± 7.4*
	4	4	99 ± 2.5
	3	10	98 ± 1.4
	3	20	102 ± 1.3
Cholesteryl acetate	5	4	97 ± 3.0
	5	10	99 ± 4.9
	4	20	99 ± 2.2

* Standard deviation.

References p. 333

DISCUSSION

The hydroxylaminolysis procedure of STERN AND SHAPIRO[1], as modified in this report, appears to be a worthwhile procedure for use in the clinical laboratory. The usefulness of cholesteryl acetate as a stable primary standard for the colorimetric analysis, avoiding the complicated standardization of olive oil, is self evident. A second factor to favor cholesteryl acetate as a standard is apparent when the absorption spectrum of the ferric-hydroxamate complexes for cholesteryl acetate, olive oil and serum extracts are compared. The spectrum for the colored complex of serum is almost identical with that of cholesteryl acetate, whereas the spectrum for the olive oil complex exhibits greater absorbance at lower wave lengths.

Variations in the absorbance maxima for the colored complex of different esters have previously been shown by other investigators[4-6]. These differences may account for the lower color values obtained for cholesteryl acetate as compared with olive oil. The data also indicate that the analysis obtained with cholesteryl acetate as the standard may represent more nearly correct values.

In sixteen essentially healthy personnel of this hospital, the fasting fatty ester concentration, as determined by the proposed procedure, ranged from 6 to 15 mmoles/l which is in good agreement with the normally accepted range of 9 to 14 mmoles/l[8]. The method, in use in this laboratory for over a year, has been a valuable adjunct in following serum fatty ester concentrations associated with altered fat metabolism in a variety of pathological entities.

SUMMARY

Crystalline cholesteryl acetate is found to be a convenient and suitable standard for the estimation of serum fatty esters by the colorimetric method. The proposed procedure has been found to be highly satisfactory for use in the routine clinical laboratory.

REFERENCES

1 I. STERN AND B. SHAPIRO, *J. Clin. Pathol.*, 6 (1953) 158.
2 S. L. TOMPSETT AND W. S. TENNANT, *Am. J. Clin. Pathol.*, 26 (1956) 1226.
3 F. LIPMANN AND L. C. TUTTLE, *Biochim. Biophys. Acta*, 4 (1950) 301.
4 R. F. GODDU, N. F. LEBLANC AND C. N. WRIGHT, *Anal. Chem.*, 27 (1955) 1251.
5 M. M. RAPPORT AND N. ALONZO, *J. Biol. Chem.*, 217 (1955) 193.
6 A. W. D. AVISON, *J. Chem. Soc.*, (1955) 732.
7 *Official Methods of Analysis of the Association of Official Agricultural Chemists*, Washington, D.C., 7th ed. (1950).
8 P. B. HAWK, B. L. OSER AND W. H. SUMMERSON, *Practical Physiological Chemistry*, 12th Ed. Blakiston Co., Philadelphia, 1947.

Received July 10th, 1958

LE TAMPON "BORATE" EN ÉLECTROPHORÈSE DE ZONES

R. HAVEZ ET G. BISERTE

Laboratoire de Chimie Biologique de la Faculté Mixte de Médecine et de Pharmacie, Lille (France)

La description de la méthode d'électrophorèse de zones sur gel d'amidon[1] a contribué très largement à l'emploi du tampon "borate". L'excellent pouvoir séparateur de cette technique a conduit à un fractionnement très poussé des globulines, différent de celui que l'on obtient sur d'autres supports (papier, amidon, gélose, etc.). Elle a permis la caractérisation de "groupes sériques"[2, 3], ainsi que la mise en évidence des divers types d'haptoglobine[4, 5]. La qualité de ces résultats s'explique en partie par la nature du gel d'amidon, qui constitue un support solide très riche en liquide, dont la porosité est bien adaptée à la séparation électrophorétique des protéines. Mais il semble également que le rôle du tampon ne soit pas négligeable et puisse expliquer partiellement la répartition obtenue. POULIK[6], par exemple, obtient des résultats plus satisfaisants en employant un tampon "tris" (tris-hydroxyméthyl-aminométhane) à l'intérieur du gel et un tampon borate dans la cuve de l'appareil à électrophorèse.

Dans la série d'expériences que nous rapportons, nous avons essayé de préciser ce rôle du tampon, – ou de la nature des ions du tampon, – en effectuant un certain nombre d'essais comparatifs en électrophorèse sur papier, en gélose et en gel d'amidon, à l'aide de deux tampons de pH identiques ou voisins, mais de composition différente.

TECHNIQUES EXPÉRIMENTALES

Les électrophorèses sur papier (papier Schleicher et Schüll No. 2043a Mgl) sont réalisées en tampon "véronal" de pH 8.9 (μ = 0.1) et en tampon "borate" de pH 8.9 (formule de SMITHIES: tampon 0.03 *M* en acide borique, 0.012 *M* en soude).

Les immuno-électrophorèses[7] ont été effectuées suivant la microméthode de SCHEIDEGGER[8]* en tampon "véronal" de pH 8.2 (μ = 0.05) et en tampon "borate" (formule de SMITHIES). La révélation des immuno-électrophorèses est faite à l'aide du sérum équin *anti-sérum humain normal* de l'Institut Pasteur de Paris.

Les électrophorèses en gel d'amidon ont été réalisées suivant la technique de MORETTI *et al.*[9] en tampon "véronal" de pH 8.9 (μ = 0.03) et en tampon "borate" (formule de SMITHIES). Deux types de révélation ont été effectués sur le gel: une coloration à l'Amidoschwarz et une révélation immunologique (voir POULIK[10]). Pour obtenir ce résultat, les coulées de gel d'amidon sont coupées longitudinalement en deux tranches égales sur toute leur longueur. La partie inférieure est colorée à l'Amidoschwarz. La partie supérieure est placée sur une lame de verre recouverte de gélose tamponnée; on creuse une gouttière dans la gélose parallèlement au grand axe de la bande rapportée de gel d'amidon, à 8 mm du bord de celle-ci, et on la remplit de sérum

* Nous tenons à remercier tout particulièrement M. le Professeur J. POLONOVSKI, Monsieur J. MORETTI et Madame G. LÉVY de leurs précieux conseils au sujet des méthodes d'électrophorèse en gélose et en gel d'amidon.

Bibliographie p. 339

équin *anti-sérum humain normal*. L'ensemble est placé dans une cuve de verre fermée saturée de vapeur d'eau et maintenue dans une pièce à température constante (21°) pendant 24 h. Finalement, la plaque de gélose est colorée à l'Amidoschwarz suivant les techniques classiques en immuno-électrophorèse (voir Fig. 1).

Dans quelques expériences, la partie supérieure de la coulée de gel d'amidon a été également découpée *dans le sens de la largeur* en différentes bandes correspondant

Fig. 1. Partie supérieure: électrophorèse en gel d'amidon (6 V/cm); révélation à l'Amidoschwarz. Partie inférieure: révélation immunologique, à l'aide d'un sérum équin *anti-sérum humain normal*, de la tranche supérieure du même gel d'amidon; noter l'étendue du trait de précipitation correspondant à la sérumalbumine (semi-schématique).

à chaque fraction protéique décelée par la coloration à l'Amidoschwarz réalisée sur la partie inférieure de la coulée de gel. Les protéines contenues dans ces bandes ont été éluées et les éluats lyophilisés ont été soumis à une immuno-électrophorèse en gélose. Cette dernière technique permet donc une étude comparative de la répartition des diverses protéines sériques au cours des deux types d'électrophorèse en gel. Une méthode identique a également été employée en partant d'électrophorégrammes séparatifs sur papier.

RÉSULTATS EXPÉRIMENTAUX

a. Électrophorèse en gel d'amidon: comparaison des répartitions protéiques en fonction de la nature du tampon

Les diagrammes d'électrophorèse en gel d'amidon réalisés soit en tampon borate, soit en tampon véronal, sont nettement différents (Fig. 2). En tampon borate, la révélation immunochimique permet de mettre en évidence plus facilement que la coloration à l'Amidoschwarz l'existence de deux préalbumines (PA_1 et PA_2) (Fig. 1). En tampon véronal de pH 8.9, on ne retrouve pas les deux préalbumines, ni le système de bandes $\alpha\beta$, dont on connaît l'importance dans la détermination des

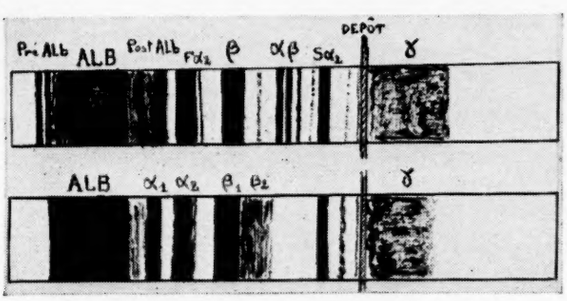


Fig. 2. Représentation schématique d'un électrophorégramme en gel d'amidon. En haut: tampon "borate" (Smithies); en bas: tampon véronal de pH 8.9, force ionique: 0.03. Les deux préalbumines et le système de bandes $\alpha\beta$ n'existent pas sur l'électrophorégramme en tampon véronal.

Bibliographie p. 339

groupes sériques. Le diagramme obtenu en gel d'amidon avec le tampon véronal présente donc une répartition assez voisine de celle que l'on obtient en électrophorèse sur papier ou sur amidon en poudre.

En vue de préciser la nature des deux préalbumines (PA_1 et PA_2), nous avons essayé de les identifier par immuno-électrophorèse en gélose (à pH 8.2) après élution des fractions séparées en gel d'amidon (tampon borate). Les résultats sont rassemblés sur la Fig. 3. L'une des préalbumines est une α_1-globuline rapide, l'autre la β_{2A}-globuline. De plus, en employant un sérum équin *anti-sérum humain normal* épuisé par l'orosomucoïde, on ne met en évidence qu'une seule préalbumine,—la plus rapide PA_1, —sur le gel d'amidon révélé immunochimiquement.

Enfin, la fraction α_1, qui existe dans les éluats de la zone des préalbumines, n'est plus révélée par le même anti-sérum épuisé par l'orosomucoïde. On peut donc, en définitive, assimiler la fraction PA_2 à l'orosomucoïde et la fraction PA_1 à la β_{2A}-globuline.

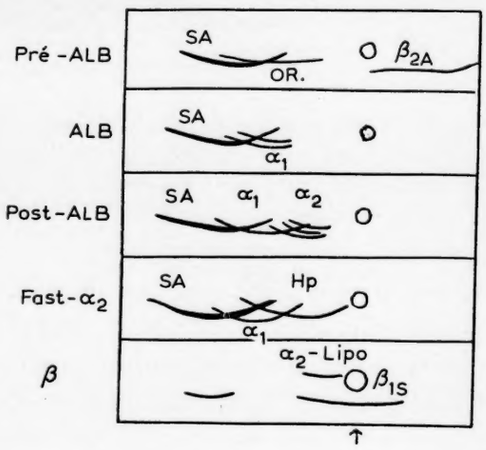


Fig. 3. Représentation schématique des immuno-électrophorèses en gélose à pH 8.2 des éluats des fractions isolées en électrophorèse en gel d'amidon (tampon "borate"). Les abréviations suivantes ont été employées: Pré-Alb = fractions préalbumines, Alb = zone de la sérumalbumine; Post-Alb = post-albumines; Fast-α_2 = α_2-globulines rapides en gel d'amidon; β = β-globulines; SA = sérumalbumine; OR = orosomucoïde; β_{2A} = β_{2A}-globuline; Hp = haptoglobine; α_1, α_2, = α_1- et α_2-globulines en immunoélectrophorèse; β_{1S} = sidérophiline; α_2-Lipo, = α_2-lipoprotéine.

En continuant l'étude de la répartition des fractions par immuno-électrophorèse en gélose des éluats des bandes successives du gel d'amidon, on trouve les post-albumines, qui correspondent à des α_1-globulines, puis trois α_2-globulines dont aucune n'est immuno-électrophorétiquement identique aux lipoprotéines. Dans la zone Fα_2 ("fast α_2"), on retrouve une fraction de vitesse intermédiaire aux α_1- et α_2-globulines et une α_2-globuline correspondant à l'haptoglobine.

La zone β du gel d'amidon donne deux traits de précipitation correspondant l'un à la β_{1S}-globuline (sidérophiline), l'autre à une α_2-globuline qui possède le comportement immuno-électrophorétique d'une lipoprotéine. Les éluats des fractions suivantes ($\alpha\beta$, Sα_2 ou "slow α_2"), qui ont été dénaturées au cours des manipulations, n'ont pas pu être entièrement identifiés: seul, un arc de précipitation de la zone α_2 reste net. Il est important de souligner que la plupart des fractions ainsi éluées contenaient toutes de la sérumalbumine. D'ailleurs, le trait de précipitation de la sérumalbumine est particulièrement allongé sur les diagrammes (voir Fig. 1), bien plus étendu que la surface colorée par l'Amidoschwarz.

b. Électrophorèse sur papier

1. *Description des diagrammes électrophorétiques en tampon borate.* Les électrophorégrammes sur papier réalisés en tampon borate sont très différents de ceux que l'on

obtient en tampon véronal de même pH (Fig. 4). Ils comprennent cinq fractions majeures.

– Une fraction préalbumine est colorable à la fois par l'Amidoschwarz et par rélévation à l'acide periodique/réactif de Schiff;

– La sérumalbumine est accompagnée d'une fraction glycoprotéique colorable par l'acide periodique/réactif de Schiff;

– Derrière la sérumalbumine, on trouve une fraction mineure d'α_1-globulines;

– La zone des α_2-globulines est relativement peu importante; elle contient également des glycoprotéines;

– La région des β-globulines est bien mise en évidence sur le protéinogramme; elle comprend deux fractions: une fraction β_1, – la plus rapide, – qui est riche en lipides et en glycoprotéines; une fraction β_2, pauvre en glycoprotéines, mais très soudanophile;

– La zone des γ-globulines est également hétérogène; au niveau des γ-globulines les plus rapides, on trouve notamment des composés soudanophiles.

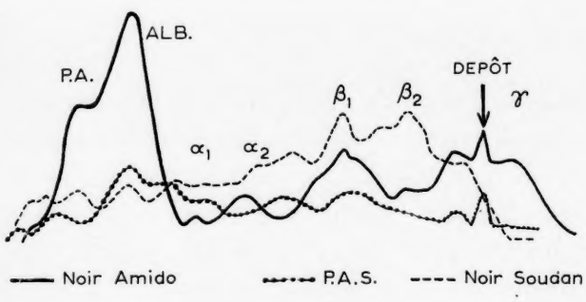


Fig. 4. Enregistrement des électrophorégrammes sur papier en tampon borate. ——— :protéinogramme (coloration à l'Amidoschwarz) ..... : glucoprotéinogramme (coloration acide periodique/réactif de Schiff) – – – – : lipoprotéinogramme (coloration au Noir Soudan).

En conclusion, les caractéristiques essentielles des électrophorégrammes sur papier en tampon borate, sont:

– l'existence d'une fraction "préalbumine" importante et la présence de glycoprotéines dans la zone préalbumine–albumine,

– l'affaissement général des α-globulines et des α-glycoprotéines,

– la dissociation des fractions lipoprotéiques dans la région des β-globulines.

Il est également intéressant de signaler que l'emploi de tampon borate produit une diminution de l'affinité tinctoriale des protéines vis-à-vis de l'Amidoschwarz; des dépôts deux fois plus importants (15 à 20 ml) de sérum sont nécessaires pour obtenir une coloration correcte.

2. *Identification immunologique des protéines séparées en électrophorèse sur papier (tampon borate).* Après une électrophorèse séparative sur papier (papier Whatman n°1, 25 × 32 cm) en tampon borate, les différentes fractions ont été étudiées en immunoélectrophorèse en gélose à pH 8.2.

La fraction préalbumine contient la β_{2A}-globuline. Dans la zone de l'albumine on trouve à la fois la sérumalbumine et l'orosomucoïde. Dans la zone immédiatement en arrière de la sérumalbumine, on retrouve une α_1- et une α_2-globuline. Dans la fraction β_1, on peut mettre en évidence la sidérophiline *.

* L'existence de l'orosomucoïde et de la sidérophiline a été démontrée soit par des réactions d'identité par la méthode de Ouchterlony, soit à l'aide de sérum équin *anti-sérum humain* épuisé par ces substances préparées à l'état pur.

Bibliographie p. 339

Les fractions les plus lentes sont dénaturées après leur élution et sont difficilement identifiées en immuno-électrophorèse.

c. Electrophorèse en gélose

Les diagrammes immuno-électrophorétiques de sérum, réalisés en gélose préparée avec du tampon borate, présentent également quelques particularités. On retrouve notamment, en avant de la sérumalbumine, une fraction nouvelle, faible dans les sérums normaux, mais nettement marquée dans les sérums de sujets présentant une affection inflammatoire comme le rhumatisme articulaire aigu. D'autre part, l'orosomucoïde se situe exactement au niveau de la sérumalbumine. Enfin, un sérum *anti-sérum humain* épuisé par l'orosomucoïde et par la sérumalbumine ne laisse subsister que la fraction "préalbumine" la plus rapide.

Le reste du diagramme immuno-électrophorétique est, par contre, assez voisin de celui que l'on obtient en tampon véronal: la α_2-, la β_{1S}- et les γ-globulines occupent leur position habituelle. La zone β_2 ne contient qu'un seul arc de précipitation.

DISCUSSION ET CONCLUSION

Cette étude comparative de deux systèmes tampons de même pH (tampon véronal et tampon borate) nous a permis de préciser les différences de répartition des protéines sériques en fonction de la nature des ions de la solution qui imbibe les différents supports d'électrophorèse de zone (papier, gel d'amidon, gélose).

Indiscutablement, l'apparition des fractions globuliniques lentes voisines du point de départ (zone $\alpha\beta$) et des Sα_2-globulines est directement en rapport avec la nature et la qualité du gel d'amidon qui doit être préparé d'une manière très rigoureuse. L'existence des préalbumines, de même que la répartition particulière des α-glycoprotéines, semble, au contraire, liée à la composition du système tampon et notamment à la présence d'ions borates. Quelle que soit la nature du support (gel d'amidon, papier, gélose), les électrophorégrammes en milieu borate contiennent des préalbumines bien distinctes. COOPER[11] a également trouvé ces fractions préalbumines en électrophorèse libre et en électrophorèse sur papier, mais il n'a pu les identifier. GARBERS ET JOUBERT[12], CONSDEN ET POWELL[13], ABDEL WAHAB *et al.*[14] ont décrit aussi des modifications comparables sur les électrophorégrammes de sérum réalisés en tampon borate.

Les deux fractions "préalbumines" sont d'ailleurs des glycoprotéines que l'on peut retrouver dans le filtrat perchlorique du sérum et qui possèdent des caractères de solubilité très particuliers et nettement différents de l'ensemble des protéines sériques. D'autre part, les interactions entre l'acide borique et les mucoprotéines ont été étudiées en électrophorèse libre par GOLDWASSER ET MATHEWS[15]: en présence de borate, les mobilités électrophorétiques de l'orosomucoïde et de l'antihyaluronidase sont augmentées. D'après les travaux de FOSTER ET STACEY[16] et ceux de NORTHCOTE[17], ces modifications peuvent s'expliquer par la formation de complexes entre l'acide borique et la copule glucidique de ces protéides.

Enfin, il est possible que la subdivision des lipoprotéines, observée en électrophorèse sur papier en tampon borate, soit en rapport avec la faible force ionique de ce tampon. SWAHN[18] a obtenu, en effet, un sous-fractionnement comparable des lipoprotéines en diminuant la force ionique du tampon véronal.

Bibliographie p. 339

RÉSUMÉ

Les auteurs ont étudié l'influence du tampon borate en électrophorèse de zones (électrophorèse sur papier, sur gélose, sur gel d'amidon). L'identification des fractions "préalbumines" a été réalisée par des techniques immunologiques: il s'agit de la β_{2A}-globuline et de l'orosomucoïde. Les modifications de vitesse des protéides sériques s'expliquent par des interactions avec les ions borates du tampon.

SUMMARY

BORATE BUFFER IN ZONE ELECTROPHORESIS

The influence of borate buffer in zone electrophoresis (on paper, gel or starch gel) has been studied. The "pre-albumin" fractions, β_{2A}-globulin and orosomucoid, have been identified by immunologic techniques. Changes in the migration speed of the serum proteins are attributable to their interaction with the borate ions of the buffer.

BIBLIOGRAPHIE

[1] O. SMITHIES, *Biochem. J.*, 61 (1955) 629.
[2] O. SMITHIES ET N. F. WALKER, *Nature*, 176 (1955) 1265.
[3] O. SMITHIES, *Nature*, 180 (1957) 1482.
[4] O. SMITHIES, *Nature*, 178 (1956) 694.
[5] J. MORETTI, G. BOUSSIER ET M. F. JAYLE, *Compt. rend. soc. biol.*, 151 (1957) 832.
[6] M. D. POULIK, *Nature*, 180 (1957) 1477.
[7] P. GRABAR ET C. A. WILLIAMS, *Biochim. Biophys. Acta*, 10 (1953) 193; 17 (1955) 67.
[8] J. J. SCHEIDEGGER, *Intern. Arch. Allergy Appl. Immunol.*, 7 (1955) 103.
[9] J. MORETTI, G. BOUSSIER ET M. F. JAYLE, *Bull. soc. chim. biol.*, 39 (1957) 593.
[10] M. D. POULIK, *Nature*, 177 (1956) 982; 180 (1957) 1477; *Can. J. Med. Sci.*, 30 (1952) 417.
[11] D. R. COOPER, *Nature*, 181 (1958) 714.
[12] C. F. GARBERS ET F. J. JOUBERT, *Nature*, 182 (1958) 530.
[13] R. CONSDEN ET M. N. POWELL, *J. Clin. Pathol.*, 8 (1955) 150.
[14] E. ABDEL WAHAB, G. ADJUTANTIS ET D. J. R. LAURENCE, *Biochem. J.*, 60 (1955) 13.
[15] E. GOLDWASSER ET M. B. MATHEWS, *J. Am. Chem. Soc.*, 77 (1955) 3135.
[16] A. B. FOSTER ET A. B. STACEY, *J. Appl. Chem. (London)*, 3 (1953) 19.
[17] D. H. NORTHCOTE, *Biochem. J.*, 58 (1954) 353.
[18] B. SWAHN, *Scand. J. Clin & Lab. Invest.*, 5 (1953) Suppl. 9.

Reçu le 10 septembre 1958

UNE CUVE À ÉLECTROPHORÈSE À USAGES MULTIPLES*

RENÉ FRENTZ

Institut de Biologie, Faculté des Sciences, Nancy (France)

L'importance de l'électrophorèse de zone n'est plus à démontrer.

L'électrophorèse sur papier de WIELAND ET FISCHER[1] est devenue une méthode de routine dans la plupart des laboratoires de recherche et de biologie clinique.

L'électrophorèse en gélose de GORDON *et al.*[2] est peut-être appelée à la remplacer progressivement car, bien que d'application plus délicate, elle présente le grand avantage d'utiliser un support transparent qui se prête bien aux évaluations photométriques.

L'immuno-électrophorèse de GRABAR ET WILLIAMS[3] combine d'une manière fort ingénieuse l'électrophorèse simple en gélose et la diffusion secondaire d'un anti-sérum dans le même milieu. Elle représente sans doute actuellement l'une des techniques les plus fines pour l'étude des protéines dans les milieux biologiques.

La micro-immuno-électrophorèse de SCHEIDEGGER[4] complète la précédente d'une manière fort heureuse, car elle en offre une variante rapide et économique qui ne met en œuvre que des quantités minimes de produit et s'adapte donc bien à des travaux préliminaires ou en séries, ou encore à des recherches sur de tout petits animaux.

Un autre support connaît aussi la faveur de nombreux laboratoires: c'est l'amidon.

L'électrophorèse dans un bloc d'amidon tamponné, de KUNKEL[5], permet la séparation de quantités importantes de substances et a déjà rendu d'appréciables services dans l'étude des hémoglobines.

L'électrophorèse en gel d'amidon de SMITHIES[6], méthode récente qu'il ne faut pas confondre avec la précédente, offre aussi de nouvelles et très intéressantes possibilités. Le support qu'elle emploie et dont les pores sont supposés être du même ordre de grandeur que les molécules de protéines à séparer, constitue une sorte de filtre sélectif qui a déjà permis d'isoler dans le sérum humain de nouvelles fractions dont l'existence et la position obéissent aux lois de la génétique. Ces premiers résultats ouvrent des horizons encore inexplorés.

Peut-être convient-il aussi de faire remarquer que si ces différentes méthodes ont donné lieu à d'innombrables recherches dans le domaine humain et celui des Mammifères, il n'en est pas de même — l'électrophorèse sur papier mise à part — pour les autres groupes zoologiques et qu'il reste là un champ d'investigation immense dont les enseignements seront des plus féconds.

Jusqu'à présent, la mise en œuvre de chacune des méthodes énumérées demandait un dispositif particulier ou une adaptation plus ou moins parfaite à des modèles existants. Ayant eu à les employer toutes, nous nous sommes demandé s'il n'était pas possible de réaliser une cuve unique permettant de les appliquer toutes, indifféremment et avec simplicité. De nombreux essais nous ont amené à construire le modèle que nous allons décrire.

---

* La cuve présentée ici et commercialement nommée "Polyphor" est construite par les Etablissements R. Chaix, 6, Avenue Milton, Nancy, France.

Bibliographie p. 345

DESCRIPTION DE LA CUVE

De forme rectangulaire, elle est construite en plexiglas transparent. Le format intérieur est 300 × 260 × 80 mm.

Une coupe schématique longitudinale est donnée par la Fig. 1.

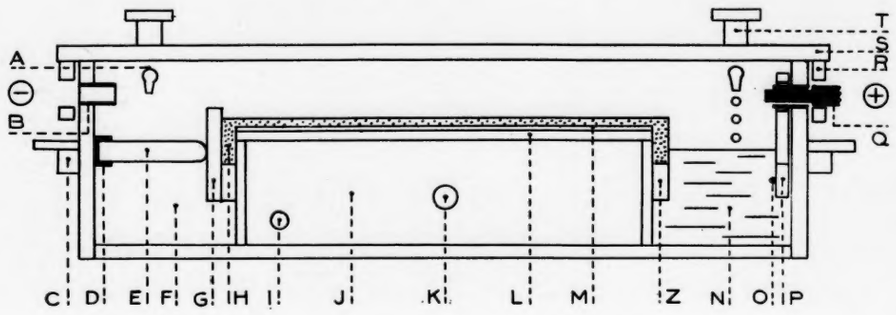


Fig. 1. Coupe longitudinale de la cuve (légende dans le texte).

Un plateau central et rigoureusement horizontal de 260 × 180 mm, en plexiglas noir (couleur destinée à faciliter les observations sur les gels) sert de table d'électrophorèse (L). Il subdivise la cuve en deux bacs latéraux (F) pour le tampon (N) et un compartiment central (J) qui permet un refroidissement par circulation d'eau (I = arrivée, K = vidange).

Sur les montants de la table sont collées deux règles (Z) dont le rôle sera précisé par la suite.

Comme dans le dispositif de GRABAR, pour éviter des variations trop importantes de pH pendant l'électrophorèse, le tampon est renouvelé goutte à goutte dans les bacs latéraux par un système d'arrivée (A) et de trop-plein (ce dernier n'étant pas visible sur les figures). Une rotation partielle des tubes de trop-plein permet le réglage du niveau de tampon; une rotation de 180° en assure la vidange automatique et totale.

Les électrodes, formées par un conducteur de platine (O) fixé sur une réglette de plexiglas (P), sont amovibles et s'adaptent par pression sur les douilles de connexion (B) qui traversent les parois latérales de la cuve.

Le couvercle (S) réalise une enceinte close et porte deux languettes (R) traversées par les fiches de connexion (Q), ce qui constitue un verrouillage de sécurité.

Cuve et couvercle portent des poignées (C, T) qui en facilitent la manipulation. Grâce aux surfaces planes et aux propriétés du plexiglas, le nettoyage de l'ensemble est des plus aisés.

Divers accessoires de conception et d'adaptation très simples permettent l'emploi de chacune des méthodes citées.

EMPLOI DE LA CUVE DANS DIVERSES MÉTHODES

Électrophorèse sur papier

Les bandes de papier filtre sont tendues dans un cadre de plexiglas (Fig. 2) qui repose en surélévation sur deux règles latérales (*U*) de la table à électrophorèse. Leurs extrémités plongent de chaque côté dans le tampon. L'électrophorèse se fait suivant les normes habituelles, comme dans n'importe quelle cuve. La surface utile

Bibliographie p. 345

maxima mesure 220 × 180 mm. Elle peut être divisée à volonté en bandes individuelles plus étroites. Les séparations obtenues sont très bonnes.

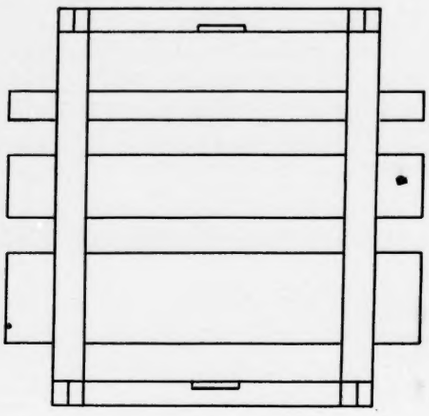


Fig. 2. Cadre-support pour l'électrophorèse sur papier portant trois bandes de largeurs différentes.

Électrophorèse en gélose

Le format des plaques utilisables peut être réglé en largeur, à volonté et sans discontinuité, jusqu'aux dimensions maxima de 240 × 180 mm.

1. *Grand format 240 × 180 mm.* La gélose tamponnée est coulée directement sur la table d'électrophorèse recouverte d'une plaque de verre de mêmes dimensions. Au-delà des bords de la table, elle tombe dans deux tranchées délimitées par les montants de celle-ci, les règles fixes (Z) et les deux plaques d'arrêt latérales (G). Ces dernières sont amovibles et temporairement maintenues par des cales (E) munies d'un embout en caoutchouc (D). Leur mise en place est des plus simples.

La gélose une fois solidifiée, cales et plaques d'arrêt sont enlevées et les retombées de gélose constituent alors une connexion directe et parfaite avec le tampon des deux bacs. Ce dispositif, dont les deux aspects successifs sont schématisés par les moitiés gauche et droite de la figure 1, évite les manipulations préliminaires des plaques et l'emploi de connexions de papier filtre. La prise du gel est notablement accélérée par le système de refroidissement. Les cuvettes de dépôt pour le mélange à séparer peuvent être faites à l'emporte-pièce ou par de petits moules en plexiglas, de taille variable, coulissant dans les rainures d'une règle-support. Ces moules, mis en place avant la coulée, s'enlèvent aisément lorsque le gel est refroidi. Leur hauteur peut être calculée pour que le fond des cuvettes reste tapissé d'une fine pellicule de gélose qui évite des fuites possibles de la substance déposée.

L'enceinte d'électrophorèse étant close et très humide, l'évaporation s'en trouve réduite, ce qui évite toute fissuration du gel surtout par temps chaud.

2. *Format variable.* Des largeurs de plaque de toutes tailles inférieures à 240 mm peuvent être réalisées sans difficulté et sans discontinuité. Il suffit de délimiter leur aire par deux ponts d'arrêt mobiles (V) dont les montants coulissent dans chacune des tranchées. La Fig. 3 concrétise cet aspect dans une cuve dégarnie de ses parois latérales et antérieure. A gauche, la plaque d'arrêt latérale est en place; à droite, elle est enlevée.

En augmentant le nombre des ponts, il devient possible de couler plusieurs bandes de gélose indépendantes pour lesquelles l'électrophorèse peut être arrêtée à des temps différents.

Bibliographie p. 345

Immuno-électrophorèse de Grabar et Williams

Après électrophorèse simple en gélose, on découpe dans le gel les cuvettes longitudinales destinées à recevoir l'immun-sérum. La plaque est alors enlevée de la cuve et la suite des opérations est conforme à la technique originale.

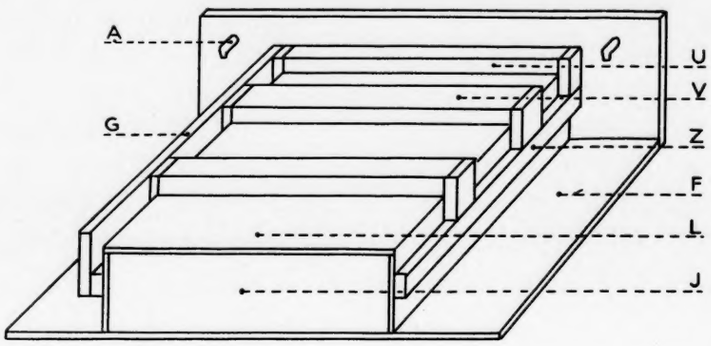


Fig. 3. Perspective de la cuve dégarnie de ses parois latérales et antérieure et montrant le système des tranchées et ponts d'arrêt.

Micro-immuno-électrophorèse de Scheidegger

Les lames d'histologie (Fig. 4: M') qui servent de support à la gélose dans cette technique sont surélevées sur la table d'électrophorèse (L) par une plaque rectangulaire de plexiglas (X) de 240 × 76 × 4 mm. La gélose liquide est arrêtée à 5 mm au-delà du bord des lames par deux règles d'arrêt (W) de 240 × 20 × 10 mm qui créent des tranchées de dimensions réduites. Cette gélose solidifiée, les règles sont enlevées et le tampon (N) amené sur le plateau par élévation correspondante des tubes de trop-plein à l'aide d'un petit embout de caoutchouc. Cet aspect est donné par la partie droite de la Fig. 4 où la règle enlevée est représentée en trait discontinu. Ici encore, la connexion électrique est directe et excellente.

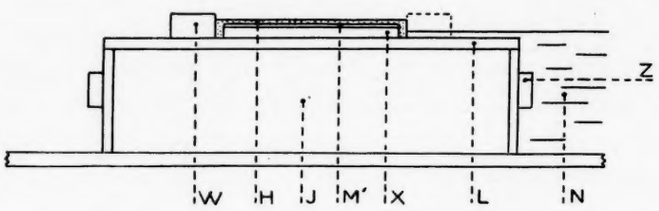


Fig. 4. Coupe transversale de la table d'électrophorèse disposée pour une micro-immuno-électrophorèse.

Les dimensions de la cuve permettent de travailler simultanément sur neuf lames porte-objet. Nous nous servons aussi de plaques de 50 × 50 mm pour diapositives qui, après séchage, coloration et montage, sont directement utilisables pour la projection.

On peut encore faire l'électrophorèse sur un nombre moindre de lames ou même sur lame unique. Il suffit alors de délimiter la surface par deux petits ponts latéraux semblables à ceux de la macrométhode et qui s'encastrent exactement sur la plaque de surélévation en coulissant dans les tranchées. L'étanchéité de la zone extérieure à ces blocs est obtenue par des joints de gélose à la pipette.

Ces dispositions, tout en conservant les possibilités de la technique originale, permettent de couler la gélose non plus individuellement sur chaque lame, mais pour

Bibliographie p. 345

l'ensemble, d'en faire varier l'épaisseur et de traiter ensuite (fixation, séchage, coloration) la totalité des lames comme un tout en se servant de la plaque de surélévation comme support.

Électrophorèse sur amidon

Les blocs d'amidon utilisés dans cette technique s'effriteraient au contact direct du tampon. Pour éviter cet inconvénient, les plaques d'arrêt de la méthode en gélose restent en position pendant toute l'électrophorèse, en laissant une ouverture à la base de la tranchée pour le passage du tampon. Ce résultat est obtenu sans accessoire supplémentaire par l'utilisation de la deuxième face des plaques. L'ouverture basale est obturée par un tampon de coton au travers duquel se fait la connexion électrique. La Fig. 5 montre le détail de cette disposition. Les dimensions possibles sont les mêmes que pour la gélose.

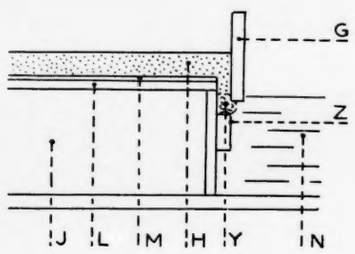


Fig. 5. Coupe transversale d'une partie de la table d'électrophorèse montrant le détail de la connexion bloc d'amidon-tampon.

Électrophorèse en gel d'amidon

La mise en œuvre de cette technique est la même que pour celle en gélose. Le gel d'amidon chaud est coulé directement sur la table d'électrophorèse recouverte d'une plaque de verre. Ici encore, le format peut être varié à volonté par simple déplacement des ponts d'arrêt et sans avoir à construire autant de moules que de formats désirés.

D'autre part, à l'essai, nous n'avons pas trouvé nécessaire de recouvrir le gel d'un couvercle comme le font les premiers auteurs. La face supérieure reste libre dans l'enceinte humide de la cuve; elle se durcit pendant le refroidissement et le passage du courant et il suffit de l'éliminer à la fin pour trouver dans l'épaisseur de la plaque une séparation excellente. Nous en avons obtenu de très fines et de très nettes, en huit heures, sans avoir à travailler en chambre froide.

De la sorte, l'électrophorèse en gel d'amidon, qui a pourtant la réputation d'un emploi délicat, nous a paru presque plus simple à appliquer que celle en gélose.

RÉSUMÉ

Est décrite une cuve à électrophorèse en plexiglas qui permet indifféremment et sans difficulté l'utilisation des six techniques suivantes: (1) Électrophorèse sur papier; (2) Électrophorèse en gélose; (3) Immuno-électrophorèse en gélose; (4) Micro-immuno-électrophorèse en gélose; (5) Électrophorèse en bloc d'amidon; (6) Électrophorèse en gel d'amidon.

Elle présente les avantages suivants: — simplicité dans l'emploi; — coulée directe des gels ou de l'amidon dans la cuve à électrophorèse, donc manipulations préli-

Bibliographie p. 345

minaires réduites; — suppression des connexions de papier filtre et contact direct du support avec le tampon; — possibilité de réaliser sans discontinuité toutes les largeurs de plaques jusqu'au format maximum de l'appareil, soit 240 × 180 mm; — électrophorèse dans une enceinte close et humide, donc évaporation réduite des gels; — possibilité de refroidissement de la table d'électrophorèse par un courant d'eau.

SUMMARY

AN ELECTROPHORESIS TANK SUITABLE FOR WIDE APPLICATION

A plexiglas electrophoresis tank is described, which can be easily used in any of the following techniques: (1) Electrophoresis on paper; (2) Electrophoresis in agar; (3) Immunoelectrophoresis in agar; (4) Micro-immunoelectrophoresis in agar; (5) Electrophoresis in slabs of starch; (6) Electrophoresis in starch gels.

This apparatus has the following advantages: It is easy to operate. The gels or starch are poured directly into the electrophoresis tank, so that preliminary manipulations are reduced. Filter paper connections are eliminated; there is direct contact between the medium and the buffer. It is possible to prepare slabs of any size up to the maximum size of the apparatus, *i.e.* 240 × 180 mm. Electrophoresis is carried out in a closed and moist chamber, thus evaporation of the gels is reduced. The electrophoresis table can be cooled with water.

BIBLIOGRAPHIE

[1] T. WIELAND ET E. FISCHER, *Naturwiss.*, 35 (1948) 29.
[2] A. H. GORDON, B. KEIL, K. SEBESTA, O. KNESSL ET F. SORM, *Collection trav. chim. tchécoslov.*, 15 (1950) 1.
[3] P. GRABAR ET C. A. WILLIAMS, *Biochim. Biophys. Acta*, 17 (1955) 67.
[4] J. J. SCHEIDEGGER, *Intern. Arch. Allergy Appl. Immunol.*, 7 (1955) 103.
[5] H. KUNKEL, in *Methods of Biochemical Analysis*, edited by D. GLICK, Vol. 1, Interscience, New York, 1954.
[6] O. SMITHIES, *Biochem. J.*, 61 (1955) 629.

Reçu le 28 octobre 1958

A RAPID MICRO-METHOD FOR DETERMINING SERUM CALCIUM

ISOBEL M. BETT* AND G. P. FRASER

Department of Clinical Chemistry, University of Aberdeen, Aberdeen (Great Britain)

Three principal methods of determining small quantities of calcium are at present in use; each has certain disadvantages when applied to the analysis of serum. The sources of error in the classical technique of KRAMER AND TISDALL[1], and its various modifications are well known. The method is tedious, requires a large volume of serum and is subject to error as a result of losses of calcium oxalate in the washing procedure. Variable amounts of magnesium are included with calcium in the estimation (MCINTYRE[2].) Large volumes of serum are necessary for determination by flame photometry, and estimation is difficult in the simpler type of flame photometer because of interference by other ions, particularly sodium and phosphate.

Many methods for the determination of calcium by titration with ethylenediaminetetra-acetic acid have been published. Direct titration of serum with EDTA using murexide as indicator (Elliot[3]) gives an unsatisfactory end-point. To overcome this difficulty the course of the titration has been followed photometrically (KIBRICK *et al.*[4], FALES[5], WILKINSON[6]).

The new indicator for the EDTA titration (calcein, fluorescene complexone) described by DIEHL AND ELLINGBOE[7] is prepared by the condensation of fluorescene and iminodiacetic acid in the presence of formaldehyde and is analogous to the phthaleins of SCHWARZENBACH (ANDEREGG *et al.*[8]). This substance in the presence of calcium in strongly alkaline solution has a green colour which changes to reddish-brown when the calcium is complexed with EDTA. Methods for the determination of calcium in serum using this indicator have been described by ANDERSCH[9] and BARON AND BELL[10].

In the present method, of which a preliminary report has been published (BETT AND FRASER[11, 12]), calcein is used as indicator and the titration carried out under ultra-violet light. The procedure is rapid, requires a small volume of serum (0.1 ml, or less if necessary), and is unaffected by interfering substances present in serum. A back titration method based on the same technique has been described by ASHBY AND ROBERTS[13].

EXPERIMENTAL

Estimation of calcium by fluorescent titration

Preparation of indicator

Calcein as prepared by the method of DIEHL AND ELLINGBOE[7] is an impure substance and all attempts to obtain the indicator in pure crystalline form have so far been unsuccessful. Chromatography on Whatman 1 paper using *n*-butanol : acetic acid : water, (100 : 22 : 50), as solvent shows the major component to be a yellow-green spot R_F 0.28. This component functions as the indicator. Some preparations and commercial samples contain a red component R_F 0.69 which fluoresces under ultra-

* Present address: Rheumatic Diseases Research Unit, Northern General Hospital, Edinburgh.

References p. 356

violet light in alkaline solution both in the presence and absence of calcium and thus obscures the end-point. This can be removed by repeated washing with absolute ethanol. The indicator is prepared as a stock solution which keeps for at least two weeks at room temperature. The working solution (4.0 mg/100 ml in 0.25 *N* NaOH) is unstable and is prepared daily. Decomposition of the indicator is shown by the persistence of fluorescence after the end-point has been reached.

Reagents

All solutions are prepared with de-ionised water and are stored in polythene.

(1) 0.25 *N* NaOH calcium free. This is prepared using polythene apparatus from a saturated solution of A.R. sodium hydroxide. (Some samples of A.R. sodium hydroxide—British Drug Houses Ltd., Poole, Dorset—contain appreciable amounts of calcium and it is necessary in the titration to make allowance for this reagent blank.)

(2) Stock calcium solution; dissolve 2.498 g dry A.R. $CaCO_3$ in 55 ml 1.0 *N* HCl and make up to 1 l with water.

(3) Working calcium standard (equivalent to 10 mg calcium/100 ml) is prepared by tenfold dilution of the stock standard.

(4) Stock solution of calcein. Dissolve 100 mg of the indicator in 10 ml 1.0 *N* NaOH and make up to 100 ml with water.

(5) Working solution of calcein, 4.0 mg/100 ml. This is prepared daily by diluting 1 ml of stock solution to 25 ml with water.

(6) Disodium ethylenediaminetetra-acetic acid solution; 2.0 g dissolved in water and made up to 1 l.

Standard procedure

Add 0.1 ml of serum or standard solution to 4.0 ml 0.25 *N* NaOH in a porcelain capsule 4 cm in diameter by 1 cm in depth. Add one drop of indicator and titrate with EDTA from a micrometer syringe under ultra-violet light. A convenient apparatus for carrying out the titration is described in the appendix. At the end-point the green fluorescence is replaced by a blue-violet reflection from the bottom of the capsule. If the 0.25 *N* NaOH is calcium free there is no blank value. This should be checked frequently, and allowance made for the blank if necessary.

Estimation of calcium by the Kramer–Tisdall procedure

The method of KING[14] with overnight precipitation of the oxalate was used.

Estimation of calcium by EDTA titration using murexide as indicator

A modification of the method of KIBRICK[4] was used. The titration was carried out in an "EEL" photoelectric colorimeter. EDTA was added from a micrometer syringe (Burroughs Welcome "Agla") and the solution stirred continuously by a fine stream of air delivered from a narrow needle reaching the bottom of the tube. A reading was taken after each addition of EDTA and the end-point determined by observation of the reading at which an addition of EDTA produced no further deflection of the galvanometer. A reagent blank was determined and subtracted from the titration figure.

References p. 356

Determination of phosphate. The method of FISKE AND SUBBAROW[15] was used.

RESULTS

Titration of standard calcium solutions

Fig. 1 shows the relationship between amount of calcium present in the capsule and volume of EDTA required to extinguish fluorescence. Amounts as small as 0.5 μg of calcium can be determined quite easily and the relationship has been found to be linear up to at least 60 μg without altering the standard conditions.

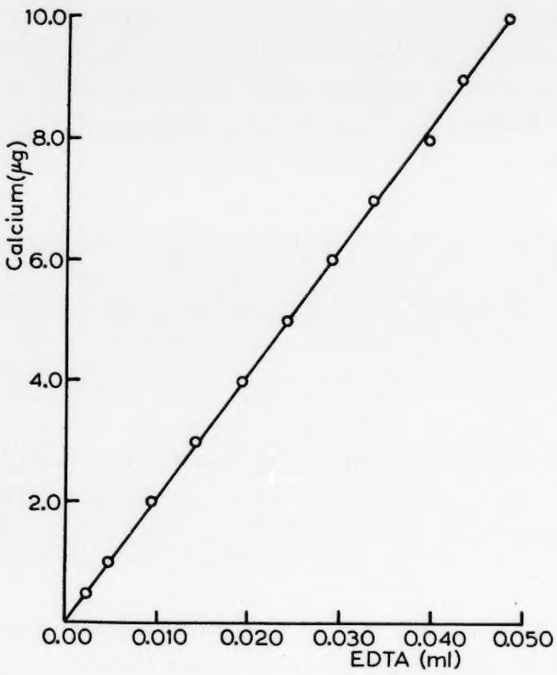


Fig. 1. Relation between amount of calcium present and volume of EDTA required to extinguish fluorescence.

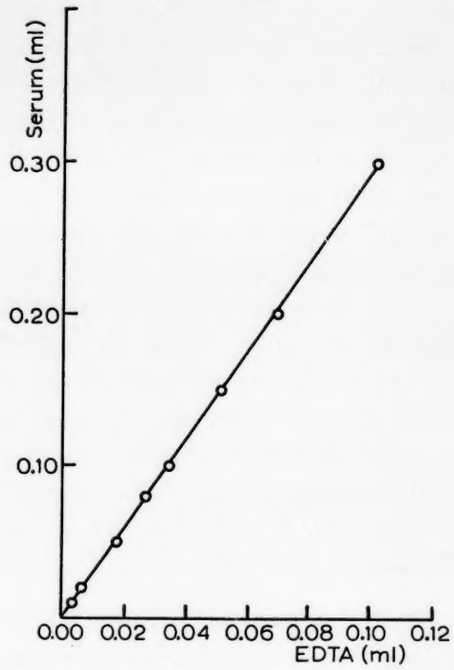


Fig. 2. Titration of different volumes of the same serum, 0.01 to 0.30 ml.

Recovery of calcium from serum

Recovery of calcium added to five human sera is shown in Table I. Although good recoveries of added calcium have been obtained, the possibility of interaction of serum components with the indicator must be considered particularly at low calcium concentration. The absence of any effect of this type is shown in Fig. 2. Different volumes of the same serum ranging from 0.01 to 0.3 ml were titrated under the same conditions and a linear relationship between serum volume and volume of EDTA obtained. Increasing the ratio of serum to indicator by a factor of 30 has thus no effect on the result. Recovery of different amounts of calcium from two samples of ox serum after ashing is shown in Table II.

The effect of magnesium and phosphate

At the pH of the titration (13) magnesium is precipitated as the hydroxide. Table III (A and B) shows recoveries of calcium in the presence of added magnesium. In Table III A, the magnesium was added to the titration dish after the serum or standard

References p. 356

TABLE I

RECOVERY OF CALCIUM FROM SERUM

Varying amounts of calcium as a solution of $CaCl_2$ containing 100 mg/100 ml Ca were added to human serum by means of a micrometer syringe or to the titration dish after the addition of the serum. Analyses in duplicate.

Serum No.	*Calcium present in serum mg/100 ml*	*Calcium added mg/100 ml*	*Total calcium mg/100 ml* (b)	*Total calcium found mg/100 ml* (a)	*(a−b) mg/100 ml*	*100 a/b*
1	8.52	0.50	9.02	8.97	−0.05	99.5
	8.52	4.35	12.87	12.94	+0.07	100.6
	8.52	8.32	16.84	16.50	−0.34	98.0
2	7.64	5.00	12.64	12.73	+0.09	100.7
	7.64	10.00	17.64	17.69	+0.05	100.2
	7.64	15.00	22.64	22.73	+0.09	100.4
3	9.66	6.00	15.66	15.62	−0.04	99.7
	9.66	10.00	19.66	19.55	−0.11	99.4
4	9.51	2.00	11.51	11.62	+0.11	100.9
	9.51	9.00	18.51	18.30	−0.21	98.9
	9.51	12.00	21.51	20.94	−0.57	97.3
5	9.72	8.00	17.72	17.84	+0.12	100.7
				Mean ± S.D.		99.7 ± 1.14

TABLE II

RECOVERY OF CALCIUM FROM OX SERUM AFTER ASHING

Varying amounts of calcium as a solution of $CaCl_2$ containing 100 mg/100 ml Ca were added to 5 or 10 ml samples of ox serum, which were then ashed overnight in platinum at 400°. The ash was digested on a water bath for 30 min with 2.0 ml 25% v/v HCl and made up to 10 ml. Duplicate analyses using 0.2 ml of digest. Values expressed as total Ca present (mg).

	Before ashing			*After ashing*	
	Serum Ca mg	*Added Ca mg*	*Total Ca (b) mg*	*Total Ca found (a) mg*	*100 a/b*
Serum 1	0.914	–	0.914	0.930	101.8
	0.914	–	0.914	0.912	99.8
	0.914	0.200	1.114	1.133	101.7
	0.914	0.500	1.414	1.400	99.0
	0.914	1.000	1.914	1.880	98.2
Serum 2	0.493	–	0.493	0.495	100.4
	0.493	–	0.493	0.503	102.0
	0.493	0.200	0.693	0.679	98.0
	0.493	0.500	0.993	0.950	95.7
	0.493	1.00	1.493	1.445	96.8
				Mean ± S.D.	99.34 ± 2.21

calcium solution had been stirred into the alkali thus giving a high local concentration of magnesium. In Table III B, the magnesium was added to the serum and the titration performed in the usual manner. No significant loss of calcium occurs at magnesium concentrations well above those encountered in serum.

The effect of added phosphate on the recovery of calcium from ox serum is shown

References p. 356

TABLE III

THE EFFECT OF ADDED MAGNESIUM

0.2 ml of serum or standard solution was stirred into the titration dish and the magnesium (as $MgSO_4$ equivalent to 1000 mg/100 ml Mg), added by means of a micrometer syringe. Analyses in duplicate.

	Equivalent concentration of added magnesium mg/100 ml	*Ca expected mg/100 ml b*	*Ca found after addition of magnesium mg/100 ml a*	*100 a/b*
Standard solution	10.0	10.00	9.88	98.8
	10.0	10.00	10.04	100.4
	20.0	10.00	9.92	99.2
	100.0	10.00	9.88	98.8
	10.0	9.92	10.00	100.8
Serum 1	20.0	9.92	9.92	100.0
	100.0	9.92	10.00	100.8
Serum 2	50.0	12.50	12.46	99.7
Serum 3	100.0	13.42	13.00	96.9

B Magnesium was added to ox serum (as $MgSO_4$ equivalent to 1000 mg/100 ml Mg) by means of a micrometer syringe and 0.2 ml used for analyses by the standard technique.

	Equivalent concentration of added magnesium mg/100 ml	*Ca expected mg/100 ml b*	*Ca found after addition of magnesium mg/100 ml a*	*100 a/b*
Serum 4	2.0	9.69	9.75	100.6
	5.0	9.66	9.77	101.1
	10.0	9.61	9.69	100.8
	20.0	9.52	9.56	100.4
Serum 5	20.0	8.95	9.05	101.1
	50.0	8.68	8.54	98.4
			Mean ± S.D.	99.85 ± 1.20

in Table IV. When high concentrations of both calcium and phosphate are present premature false end-points are obtained; the green fluorescence disappears and returns after stirring for a few minutes. When standard solutions of calcium containing phosphate were titrated no clear cut concentration at which false end-points were obtained could be found. However, no fading was observed in solutions containing less than 10 mg/100 ml Ca and 10 mg/100 ml P (Table V A). That the effect is not due to interference by the phosphate ion *per se* was shown by experiments in which a standard solution of the two ions was added to 3 ml of water in the titration dish and 1 ml of 1 *N* NaOH added with stirring to give the same final concentration of alkali. No false end-points were obtained in solutions containing up to 40 mg/100 ml Ca and 30 mg/100 ml P, whatever the ratio of the two ions (Table V B).

Recovery from pigmented sera. A moderate degree of haemolysis has little effect on the titration (Table VI) other than to cause a whitish fluorescence at the end-point. Grossly haemolysed sera are difficult to titrate because of the fluorescence at the end-

References p. 356

TABLE IV

THE EFFECT OF PHOSPHATE ON THE RECOVERY OF CALCIUM FROM OX SERUM

Phosphate (as KH_2PO_4 solution equivalent to 100 mg/100 ml P) was added in varying amount to three ox sera from a micrometer syringe. Analyses in duplicate.

	Phosphorus mg/100 ml P	*Ca expected mg/100 ml* *b*	*Ca found after addition of phosphorus mg/100 ml* *a*	*100 a/b*
Serum 1	5.5	–	9.11	–
	8.0	9.08	9.11	100.3
	10.0	9.06	9.07	100.1
	11.5	9.05	9.09	100.4
	13.0	9.03	9.09	100.7
	19.0	8.97	9.00	100.3
Serum 2	7.1	–	9.44	–
	7.1	13.97	13.85	99.1
	8.2	13.94	13.94	100.0
	11.2	13.90	13.90	100.0
	13.5	13.87	13.81	99.6
	16.0	13.83	13.65	98.7
Serum 3	6.6	–	–	–
	8.5	19.36	19.26	99.5
	10.2	19.30	19.11	99.0
	12.2	19.26	18.93	98.3*
	16.0	19.20	18.80	97.9*
	22.4	19.01	18.35	96.5*
			Mean ± S.D.	99.81 ± 0.73

* False end point; not included in mean value.

TABLE V

THE EFFECT OF PHOSPHATE ON THE TITRATION OF AQUEOUS CALCIUM STANDARDS

Normal end-point . . . . . +
Slight fading at the end-point with return of fluorescence . . . . . s.f.
False end-point with incorrect result . . . . . f.e.p.

A Solutions of calcium containing phosphate (as KH_2PO_4) were titrated by the standard technique.

		Ca (mg/100 ml)			
		5	10	15	20
P (mg/100 ml)	5	+	+	s.f	f.e.p.
	10	+	+	s.f.	f.e.p.
	15	+	s.f.	s.f.	f.e.p.
	20	+	s.f.	f.e.p.	f.e.p.

B 0.1 ml of standard calcium solution containing phosphate was added to 3 ml water and 1 ml 1.0 *N* NaOH added with stirring giving the same final concentration of NaOH as in A.

		Ca (mg/100 ml)				
		10	20	30	40	50
P (mg/100 ml)	10	+	+	+	+	+
	20	+	+	+	+	+
	30	+	+	+	+	s.f.
	40	+	+	+	f.e.p.	f.e.p.
	50	+	+	f.e.p.	f.e.p.	f.e.p.

References p. 356

TABLE VI

RECOVERY OF CALCIUM IN THE PRESENCE OF PIGMENTED SERUM

Different amounts of calcium (as a solution of $CaCl_2$ containing 100 mg/100 ml) were added to the titration dish after the addition of 0.2 ml of serum.
Analyses in duplicate

Serum No.	*Ca obtained mg/100 ml a*	*Ca expected mg/100 ml b*	*a—b*	*100 a/b*
	(a) 8.93	–	–	–
haemolysed	(b) 13.96	13.93	+0.03	100.2
	(c) 18.90	18.93	−0.03	99.8
grossly	(a) 8.98	–	–	–
haemolysed	(b) 16.43	16.98	−0.55	96.7
	(a) 7.93	–	–	–
bilirubin	(b) 12.06	11.93	+0.13	101.1
3 mg/100 ml	(c) 15.84	15.93	−0.09	99.5
bilirubin	(a) 8.46	–	–	–
8 mg/100 ml	(b) 18.35	18.46	−0.11	99.4
bilirubin	(a) 9.66	–	–	–
17 mg/100 ml	(b) 14.90	14.66	+0.24	101.1
		Mean ± S.D.		99.8 ± 1.58

point but this can usually be observed at a second titration. A similar effect is observed in icteric sera where a yellow fluorescence tends to obscure the end-point. The effect does not cause serious interference until the bilirubin concentration rises to above about 10 mg/100 ml. With more severely jaundiced sera the end-point can usually be observed from a second titration.

Comparison of results obtained by the KRAMER-TISDAL, *murexide titration and ultra-violet titration techniques.*

The mean results for 30 sera analysed by the three techniques are shown in Table

TABLE VII

COMPARISON OF RESULTS OBTAINED BY THE THREE METHODS

Source of sera	*No. of sera*	*Mean (mg/100 ml) ± S.D.*		*Mean difference*	*S.E.M.*
Blood donors (sample taken at end of bleeding period)	30	*Ultra-violet titration* 9.46 ± 0.23	KRAMER–TISDALL 9.38 ± 0.38	0.08	0.045
Normal and pathological sera (obtained by venepuncture)	52	*Ultra-violet titration* 9.46 ± 0.23	*Murexide titration* 9.49 ± 0.36	0.03	0.045
		Murexide titration 9.65 ± 1.02	KRAMER–TISDALL 9.59 ± 1.02	0.06	0.042
Normal sera (obtained by venepuncture)	24	*Ultra-violet titration* 9.90 ± 0.41		–	–

References p. 356

VII. Although the mean result for the ultra-violet titration is higher than that for the KRAMER-TISDALL technique, the difference is not significant. The means for the ultra-violet and murexide titrations were also not significantly different. When 52 normal and pathological sera were analysed by the murexide and KRAMER-TISDALL procedures no significant difference between the means was obtained.

The error of the method. Analysis of a single serum by the ultraviolet titration technique gave a mean result of 7.64 mg/100 ml $\pm$ 0.045 (S.E.M.; 10 estimations).

Duplicate estimations on 25 normal sera gave a mean difference between duplicates of 0.08 $\pm$ 0.047 (S.E.M.), the precision $\sqrt{(\Sigma d^2/2n)}$ being 0.066.

DISCUSSION

Although murexide is a specific indicator for the complexometric titration of calcium by EDTA, the technique is difficult to adapt for small volumes of serum because of the gradual colour change at the end-point. This difficulty can be partially overcome by carrying out the titration in a photometer or spectrophotometer which entails finding the end-point graphically (WILKINSON[6]) or by recording a series of readings as in the method described here in the experimental section. Visual methods using calcein as indicator give improved end-points particularly when the indicator is screened with thymolphthalein (TUCKER[16], BARON AND BELL[10]). It has been found, however, that the endpoint is more easily determined under ultra-violet light, giving a considerable improvement in the precision of the method. The greater sensitivity of the fluorescence technique allows very small amounts (0.5 μg in 3 ml) to be estimated successfully.

The reliability of the method has been investigated by adding known amounts of calcium to serum: good recoveries of added calcium are obtained (Table I). Results obtained after ashing the serum correspond well with those given by direct titration (Table II).

The effect of interfering substances.

SCHWARZENBACH[17] has reported that calcium is co-precipitated with magnesium as the hydroxide at pH 13. No significant fall in the recovery of calcium has been found when magnesium is added to serum in many times the normal concentration, (Table III A, B) and it must be concluded that with efficient stirring and at the dilution of the titration this effect does not operate.

With solutions containing high concentrations of both calcium and phosphate premature end-points and low recoveries are obtained. The results in Table IV show that it is unlikely that concentrations of both ions high enough to cause this effect could be present in any pathological serum. False end-points are obtained with urine, however, and this effect along with the strong fluorescence of some samples makes the method unsuitable for untreated urines. COLLIER[18] has studied the effect of phosphate on the complexometric titration of calcium using murexide as indicator and states that there is no definite ratio of phosphate to calcium at which interference begins but that it increases gradually with concentration and there is an inverse ratio between the calcium concentration and the maximum permissable phosphate concentration.

The results obtained with calcein confirm these observations. With aqueous solutions false end-points are obtained when the concentration of calcium and phosphate

References p. 356

is above approximately 10 mg/100 ml for each ion (Table VA). With serum these concentrations can be considerably exceeded before fading occurs (Table IV). If the standard solution containing calcium and phosphate is diluted with water before the addition of alkali, the concentrations of calcium and phosphate can be raised considerably before false end-points result (Table V B). It appears, therefore, that the phosphate ion does not interfere with the titration, but if a high enough concentration of calcium and phosphate is present precipitation occurs as the solution is added to the alkali and false end-points are obtained. Serum appears to retard the precipitation of calcium phosphate.

Sera from uraemic patients occasionally show a bright bluish white fluorescence which persists throughout the titration and tends to obscure the end-point. The change is from bluish white to violet and can be observed quite easily with experience. Sera which have been standing at room temperature for a day or two occasionally show a strong green fluorescence which completely obscures the end-point. These sera have always been found to be contaminated with *Pseudomonas fluorescens*, whose fluorescence is very similar to that of calcein.

Sera from patients receiving large doses of salicylates show a strong blue-white fluorescence. The end-point of the titration is extremely sharp and occurs as a change from blue-white to deep violet fluorescence. This has been shown to be the true end-point by recovery experiments.

Comparison of results obtained by the three methods

The results in Table VII show that in our hands there is no significant difference between the means obtained in the ultra-violet titration, murexide titration and the KRAMER–TISDALL technique. The sera used in this experiment were obtained from normal blood donors and were collected at the end of the bleeding period. The mean values are considerably lower than those of WOOTTON, KING AND MACLEAN SMITH[19] for normal adults and are not the true normal values for the three methods. Sera taken by venepuncture using a 5-ml syringe in the usual manner from 24 normal adults were analysed by ultra-violet titration and gave a mean result of 9.90 mg/100 ml $\pm$ 0.41. The investigation of this marked discrepancy will be reported later.

APPENDIX

An Ultra-Violet Titrator

The performance of titrations in ultra-violet light under normal laboratory conditions presents several difficulties particularly where darkroom facilities are not available. In addition to excluding daylight and protecting the eye of the observer, this apparatus provides a convenient means of stirring and adding increments of titrant.

The apparatus is illustrated in Fig. 3; the dimensions are not critical. The body of the instrument is a tin-plate cylinder 36 cm long by 11 cm in diam., the upper part of which forms the lamphouse containing a low pressure mercury arc source (a), (Philips MBL/U 125 W with high emission at 3650 Å). Visible light is excluded by a Wood's glass filter (Ilford 828), (b). The base of the titration chamber consists of a magnetic stirrer (c), which can be racked down to admit the titration dish and then raised into the body of the instrument to exclude light. The titration dish is observed

References p. 356

through a plain tube (d), fitted with face shield and set at an angle to the titration chamber. An Ilford 805 filter (e), in the lower part of this tube protects the eyes from stray ultra-violet light. The inner surfaces are painted matt black.

The titrant is added by means of a micrometer syringe (f), (Burroughs Welcome "Agla") fitted with a polythene needle reduced at the tip to approximately 0.1 mm in diam. The needle passes into the titration chamber through a velvet lined slit (g), and is so arranged that the tip of the needle dips just below the surface of the solution when the magnetic stirrer is raised. The position of the needle can easily be found because of its fluorescence. The micrometer syringe is refilled by placing a small beaker of EDTA on the magnetic stirrer, raising the stirrer and withdrawing the plunger.

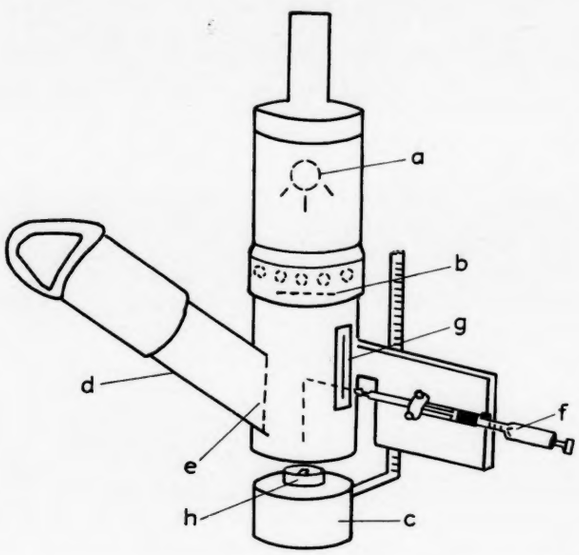


Fig. 3. Ultra-violet titration apparatus: (a) Ultra-violet light source (Philips MBL/U 125 W); (b) Wood's glass filter; (c) Magnetic stirrer; (d) Eyepiece; (e) Light filter (Ilford 805); (f) Micrometer syringe; (g) Slit for micrometer needle; (h) Porcelain capsule.

The sharpness of the end-point is influenced by the type of titration dish used; the most satisfactory has been found to be small porcelain capsule (h), 4 cm in diameter and 1 cm in depth. Magnetic followers enclosed in opal "Perspex" are non-fluorescent and are more durable than glass. Increasing the depth of the solution does not improve the sensitivity of the method but the diameter of the dish may be reduced considerably and the volume of the solution adjusted when very small amounts of calcium are being estimated.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Dr. A. LYALL and members of the staff of the Clinical Chemistry Department for interest shown in this work.

They also wish to thank student volunteers and Dr. J. CAMPBELL of the North East of Scotland Blood Transfusion Service for supplying normal blood samples.

SUMMARY

1. A micro method for the determination of calcium in serum by direct titration under ultra-violet light with EDTA, using the fluorescent indicator calcein, is describ-

References p. 356

ed. Good recoveries of calcium from serum, both directly and after ashing, have been obtained.

2. The effect of possible interfering substances present in serum has been investigated. Magnesium and phosphate do not interfere in the concentrations present in serum and the determination can be made on haemolysed and icteric sera.

3. Thirty sera have been analysed by fluorescent titration, murexide titration and by the KRAMER–TISDALL technique. No significant difference has been found between the results given by the three methods.

4. A convenient apparatus for carrying out titrations under ultra-violet light has been described.

REFERENCES

1 B. KRAMER AND F. TISDALL, *J. Biol. Chem.*, 46 (1921) 339.
2 I. MACINTYRE, *Biochem. J.*, 67 (1957) 164.
3 W. E. ELLIOT, *J. Biol. Chem.*, 197 (1952) 641.
4 A. C. KIBRICK, M. ROSS AND H. E. ROGERS, *Proc. Soc. Exptl. Biol. Med.*, 81 (1952) 353.
5 F. W. FALES, *J. Biol. Chem.*, 204 (1953) 577.
6 R. H. WILKINSON, *J. Clin. Pathol.*, 10 (1957) 126.
7 H. DIEHL, AND J. L. ELLINGBOE, *Anal. Chem.*, 28 (1956) 5, 881.
8 G. ANDEREGG, H. FLASCHKA, R. SALLMANN AND G. SCHWARZENBACH, *Helv. Chim. Acta*, 37 (1954) 113.
9 M. A. ANDERSCH, *J. Lab. Clin. Med.*, 49 (1957) 3, 486.
10 D. N. BARON AND J. L. BELL, *Clin. Chim. Acta*, 2 (1957) 327.
11 I. M. BETT AND G. P. FRASER, *Biochem. J.*, 68 (1958) 2, 13p.
12 I. M. BETT AND G. P. FRASER, *Biochem. J.*, 68 (1958) 2, 20p.
13 R. O. ASHBY AND M. ROBERTS, *J. Lab. Clin. Med.*, 49, (1957) 6, 958.
14 E. J. KING, in *Micro-Analysis in Medical Biochemistry*, 2nd ed., J. and A. Churchill Ltd., London, 1951, p. 84.
15 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
16 B. M. TUCKER, *Analyst*, 82 (1957) 284.
17 G. SCHWARZENBACH, in *Complexometric Titrations*, translated and revised by H. IRVING, Methuen & Co. Ltd., London, 1957, p. 23.
18 R. E. COLLIER, *Chem. & Ind. (London)*, (1955) 587.
19 I. D. P. WOOTTON, E. J. KING AND J. MACLEAN SMITH, *Brit. Med. Bull.*, 7 (1951) 4, 307.

Received October 30th 1958

ULTRA-MICRODOSAGE AUTOMATIQUE DU CALCIUM SERIQUE

P. CARTIER ET J. CLÉMENT-MÉTRAL

Laboratoire d'Étude de l'Os et de la Croissance, Faculté de Médecine, Paris (France)

Établir avec rapidité et précision un ionogramme complet sur un faible volume de sérum (en particulier chez le jeune enfant), est un problème qui se pose fréquemment. Or, si le sodium et le potassium sont dosables rapidement et sur de faibles volumes de sérum, par photométrie de flamme, le calcium et le magnésium nécessitent habituellement des prélèvements plus importants et des techniques délicates et longues, qui excluent de l'ionogramme, souvent demandé d'urgence, le dosage de ces deux éléments. Un autre problème faisant intervenir les mêmes considérations s'est posé à nous dans le domaine de la recherche: la mesure de l'activité de préparations parathyroïdiennes, par la détermination des variations de la calcémie du rat thyro-parathyroïdectomisé.

Le dosage du calcium sérique par précipitation sous forme d'oxalate après calcination, et dosage manganimétrique du précipité, constitue toujours la méthode de référence[1, 2]. Mais cette technique est laborieuse et nécessite un volume important de sérum (2 à 4 ml). La photométrie de flamme n'est pas habituellement employée, en raison surtout de son manque de sensibilité et de spécificité, qui entraine des corrections de mesure difficiles. Récemment, l'application à la micro-détermination du calcium sanguin des propriétés complexantes de l'EDTA (acide éthylène-diamine-tétraacétique), ont représenté un réel progrès de simplicité et de rapidité. Les nombreuses techniques proposées[3–5] reposent sur la propriété de l'EDTA de complexer tout le calcium présent dans le sérum (sous quelque forme qu'il soit), dans un rapport équimoléculaire. La fin de la titration est appréciée par le virage d'un indicateur convenablement choisi qui change de teinte en présence ou en l'absence d'ions Ca^{+2} libres. Les plus fréquemment employés de ces indicateurs sont:

a. le *Noir d'Eriochrome T*, qui est stable, possède un virage franc, mais dose à la fois calcium et magnésium: de ce fait, le dosage séparé du magnésium devient obligatoire et rend la méthode inutilisable pour le dosage direct de la calcémie[6, 7];

b. la *Murexide*, qui, dans des conditions de pH déterminées, est spécifique du calcium (pH 12 environ). Mais cet indicateur présente de multiples inconvénients. Les maxima d'absorption des deux formes (libre et complexée) sont suffisamment décalés, mais le virage d'une forme à l'autre est progressif et très difficile à apprécier à l'œil. Cette imprécision du virage est améliorée par l'adjonction d'un colorant de contraste[8] et surtout par la mesure photométrique du virage. Nous avons proposé un microdosage automatique dérivé de ce principe[9], dans lequel nous enregistrons la courbe de virage à une longueur d'onde où la différence de densité optique des deux formes est la plus marquée, au pH utilisé. Cependant, nous avons abandonné cette technique en raison de l'instabilité de la murexide en solution. En effet, selon différents auteurs[10–13], et d'après notre expérience personnelle, la couleur n'est pas stable plus de 15 min et la solution de l'indicateur commence à se décomposer en 2 à 3 h. Cette coloration est également sensible aux variations de pH et l'emploi de la murexide à l'état solide

Bibliographie p. 363

(habituellement diluée dans du chlorure de sodium pulvérisé), proposé par BUCKLEY[14–16] ne permet pas d'ajouter une quantité constante d'indicateur, à moins de s'astreindre à peser le mélange avant chaque dosage.

c. Récemment, DIEHL ET ELLINGBOE[17] ont décrit un nouvel indicateur, produit de condensation de la fluorescéine et de l'acide imino-diacétique, qu'ils ont appelé *"Calcéine"*, spécifique du calcium. Cet indicateur, d'une remarquable stabilité, vire du jaune-vert au brun à pH supérieur à 12 quand tout le calcium de la solution est complexé. En réalité, le virage de la calcéine est également difficile à apprécier visuellement. TUCKER[18] et BARON[19] ont préconisé l'addition d'un colorant (thymolphtaléine) pour accuser le contraste du virage, mais soulignent que des traces d'hémolyse modifient les colorations. Toutefois, il semble que la préparation de calcéine de DIEHL ET ELLINGBOE constitue un mélange et KÖRBL ET VYDRA[20] ont réalisé la condensation de la fluorescéine et de l'acide imino-diacétique selon une autre méthode: la calcéine ainsi préparée ne présenterait plus de virage dans les conditions du dosage complexiométrique, mais une intense fluorescence qui disparait en l'absence d'ions Ca^{++}; ce phénomène avait déjà été signalé[21, 22].

C'est cette propriété de la calcéine que nous avons appliquée au microdosage du calcium sérique, que nous présentons dans ce travail, et qui nous permet d'effectuer avec une excellente précision le dosage direct du calcium sur un échantillon de sérum inférieur à 0.1 ml.

TECHNIQUE

1. *Réactifs*

Toutes les solutions sont préparées à l'eau bi-distillée et conservées dans des flacons en polyéthylène. Les précautions requises dans les microdosages[23] sont évidemment respectées et la verrerie, lavée au mélange sulfo-chromique, est rincée à l'eau bi-distillée.

NaOH 0.25 *N* (Merck purissime)

Solution étalon de calcium à 100μg/ml. (Dissoudre 0.2497 g de $CaCO_3$)R.P.), séché 24 h à 100°, dans un peu d'HCl (R.P.) redistillé. Chasser l'acide et le CO_2 à l'ébullition. Après refroidissement, amener à un litre avec de l'eau bi-distillée). Le titre est vérifié par la méthode de CLARK ET COLLIP.

Solution-mère d'EDTA 0.1 *M*: Dissoudre 37.21 g de Complexon III (sel disodique de l'acide ethylènediamine-tétra-acétique) dans environ 600 ml d'eau. La solution est amenée à pH 10.5–11 avec NaOH *N* et complétée à 1000 ml avec de l'eau bi-distillée.

Solution d'EDTA 0.01 *M*: Dilution au 1/10^e de la solution-mère.

*Calcéine**: Solution-mère préparée selon ANDERSCH[24]. Dissoudre 2 g de Calcéine dans 25 ml de NaOH *N* et compléter à 250 ml avec de l'eau bi-distillée. Nous utilisons une dilution dans l'eau bi-distillée au 1/100, que nous préférons préparer chaque jour (bien qu'elle soit stable pendant plusieurs jours).

Anti-moussant: une solution d'octanol secondaire à 20% dans l'alcool éthylique à 95%.

---

* G. Frederick Smith Chemical Co., Colombus, Ohio, U.S.A.

Bibliographie p. 363

2. *Matériel*

Micropipettes de 10 à 40 μl ou micropipettes automatiques du type LANG-LEVY (20 à 100 μl). Tubes de polyéthylène de 4 mm de diamètre extérieur, baguettes de verre de même diamètre (utilisés en cas de micro-prélèvements chez le Rat). Fluorescimètre Photovolt Corporation (New York City), équipé d'un écran primaire (Pr = Hg 1–2–3) et d'un écran secondaire interférentiel (Sec = 520 mμ). Suiveur de Spot Graphi-Spot Sefram relié au fluorescimètre. Pompe à aquarium, qui assure l'agitation dans la cuve par l'intermédiaire d'une aiguille métallique. Seringue micrométrique AGLA (Burroughs Wellcome and Co., Londres) dont le piston, entrainé par un micromoteur*, est solidaire d'un contact qui inscrit les tours du palmer et par conséquent les volumes d'EDTA versés (Fig. 1). La seringue est reliée par un fin tube en polyéthylène (diamètre 1 mm), à un tube capillaire qui plonge dans la cuve de mesure (cuve de verre à 4 faces transparentes 1 cm × 1 cm). Le capillaire est solidaire de l'aiguille d'agitation (Fig. 1).

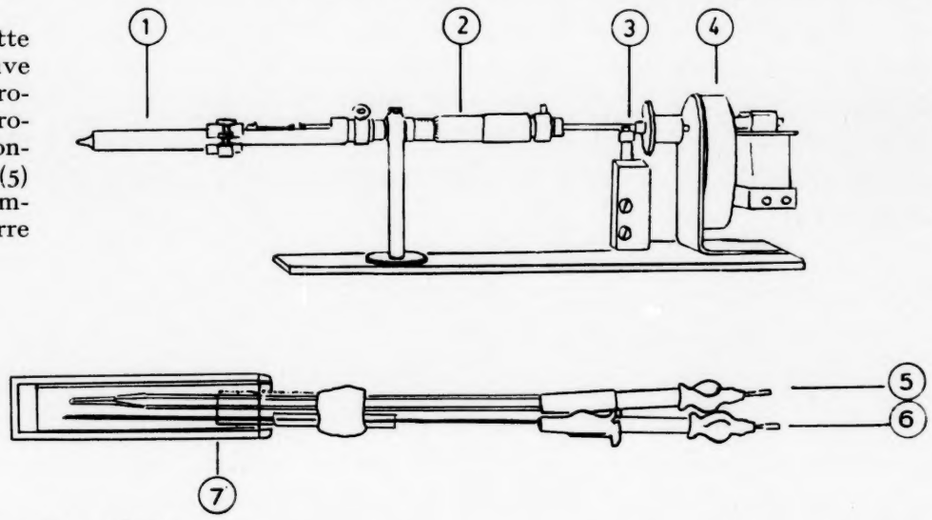


Fig. 1. Microburette automatique et cuve de mesure: (1) microseringue, (2) micromètre AGLA, (3) contact, (4) moteur, (5) capillaire, (6) air comprimé, (7) cuve verre 1 × 1 cm.

3. *Prélèvement du sang (Rat)*

Le sang est prélevé par section de la queue et immédiatement introduit dans une portion de tube de polyéthylène dont l'extrémité a été évasée. On recueille environ 0.5 ml et on ferme avec un petit morceau de baguette de verre l'extrémité inférieure. On centrifuge immédiatement. On aspire ensuite avec une pipette PASTEUR un volume de plasma que l'on dépose sur une plaque à godets vaselinée. Très rapidement, pour éviter l'évaporation, on prélève avec une micropipette la quantité désirée et on effectue le dosage.

4. *Dosage*

La courbe de titrage du calcium (introduit dans un grand volume de NaOH 0.25 *N* en présence de Calcéine trés diluée), par une solution d'EDTA di-sodique 0.01 *M* (préalablement titrée par une solution étalon de $CaCl_2$ à 100 γg/ml dans les mêmes conditions), est enregistrée continuement à l'aide du suiveur de spot "Graphi-Spot" relié au fluorescimètre (vitesse: 60 mm/min). L'aiguille amenant l'air comprimé plonge au fond de la cuve et le biseau est tourné vers le capillaire de la burette. Le débit est réglé de manière à obtenir une agitation vigoureuse pour mélanger instantanément l'EDTA versé: les bulles d'air ne gênent habituellement pas l'enregistrement.

* Sapmi, un tour/minute, sens inversé.

Bibliographie p. 363

En raison de la présence dans la soude, même purissime, de traces de calcium que la sensibilité de la méthode rend décelables, on détermine la quantité d'EDTA nécessaire pour faire virer la calcéine en présence de soude (soit 3 ml NaOH 0.25 *N* et 50 μl de solution diluée de calcéine).

L'étalonnage de l'EDTA est effectué en introduisant de 20 à 100 μl de la solution étalon de calcium. Le dosage du calcium sérique est réalisé dans les mêmes conditions sur une prise d'essai de 20 à 100 μl (selon le volume disponible) en présence de quelques gouttes d'anti-moussant. Les dosages sont effectués en double.

5. *Calculs*

On obtient ainsi les trois courbes de titrage correspondant à: témoin, étalon et sérum à doser.

Le début du titrage est donné par l'inscription du signal qui est déclanché en même temps que la rotation du moteur de la microburette; la fin par l'intersection entre la partie ascendante et le plateau de la courbe. Le volume d'EDTA utilisé pour complexer le calcium est donné par l'abscisse de la courbe que l'on peut mesurer avec précision.

Soient *T* (témoin), *E* (Ca étalon), *S* (sérum), les volumes d'EDTA versés (ou la valeur des absicsses en mm), la teneur en Ca dans la prise d'essai (exprimée en μg) est donnée par l'expression:

$$\frac{S - T}{E - T} \times \mu\text{g Ca dans } E$$

RÉSULTATS

La pente de la courbe peut être modifiée en agissant soit sur la sensibilité du galvanomètre, soit sur l'intensité de la lumière d'excitation de fluorescence. La Fig. 2

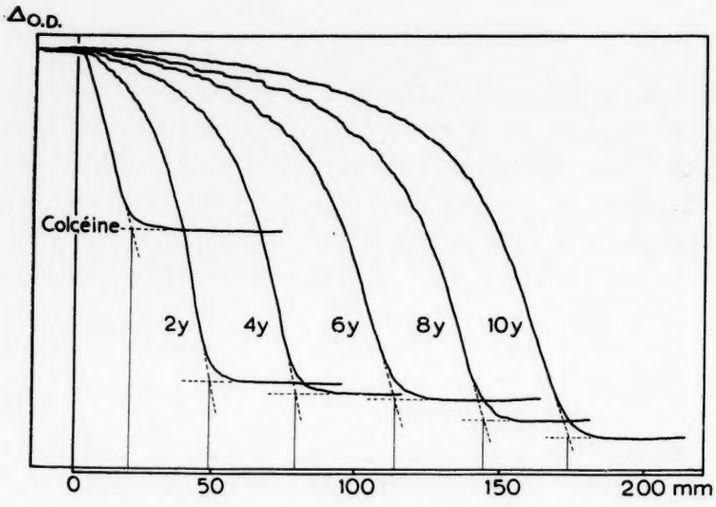


Fig. 2. Enregistrements des titrages de 5 concentrations croissantes d'une solution étalon de calcium (100 μg/ml) par l'EDTA 0.01 *M*.

représente les enregistrements de 5 concentrations différentes de calcium étalon. La Fig. 3 montre la proportionnalité parfaite entre les volumes d'EDTA versés et les concentrations en calcium (rapport 1/1 du complexe EDTA–calcium). Nous l'avons vérifiée sur vingt courbes d'étalonnage. Sur la courbe supérieure chaque mesure est figurée avec sa déviation standard. La courbe inférieure est déduite de la précédente en soustrayant la valeur du témoin.

Bibliographie p. 363

Le Tableau I, qui rassemble les valeurs des calcémies dosées par la technique proposée et comparées à celles obtenues par la méthode de CLARK ET COLLIP (moyenne

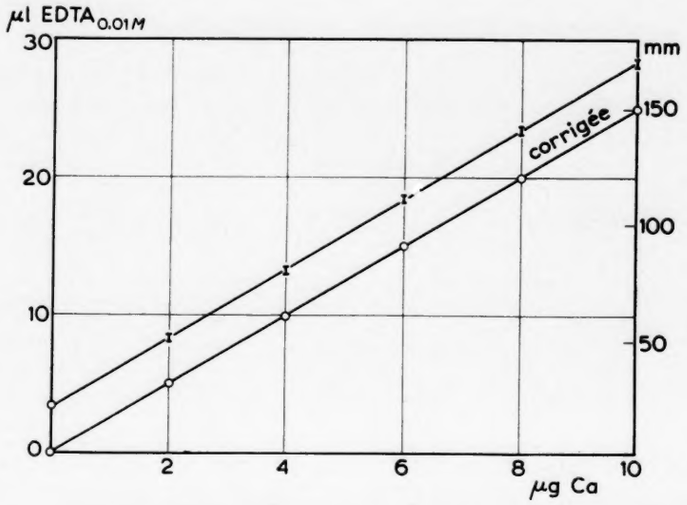


Fig. 3. Courbes d'étalonnage.

de deux dosages), montre que les valeurs trouvées par fluorimétrie ne diffèrent pas de plus de 2% de celles fournies par la méthode classique de CLARK ET COLLIP.

TABLEAU I

COMPARAISON DES CALCÉMIES (EXPRIMÉES EN mg/l) DÉTERMINÉES PAR LA TECHNIQUE FLUORIMÉTRIQUE ET PAR LA MÉTHODE DE CLARK ET COLLIP

N°	*Fluorimétrie*	CLARK ET COLLIP	*Différence*	
			val. abs.	%
1	100.0	99.9	0.1	+ 0.1
2	97.4	96.7	0.7	+ 1
3	106.0	106.4	0.4	— 0.4
4	96.0	96.0	0.0	0
5	96.0	95.0	1.0	+ 1
6	104.0	102.0	2.0	+ 2
7	100.4	100.0	0.4	+ 0.4
8	97.0	99.0	2.0	— 2
9	105.0	104.0	1.0	+ 1
10	96.0	95.5	0.5	+ 0.5
11	111.0	113.0	2.0	— 2
12	94.0	94.0	0.0	0
13	86.0	85.5	0.5	+ 0.5
			moyenne =	± 0.8%

DISCUSSION

Sur 10 échantillons de 2 concentrations de la solution étalon de calcium, nous avons effectué le microdosage et calculé la déviation standard des résultats selon la formule:

$$\sigma = \sqrt{\frac{\Sigma(x - m)^2}{n - 1}}$$

(où x est la valeur du dosage, m la moyenne de ces valeurs et n leur nombre), et les erreurs standard sur la moyenne et la différence des moyennes par les formules:

Bibliographie p. 363

$$\text{S.E.M.} = \frac{\sigma}{n} \text{ et S.E.}_{\text{Diff. des } m} = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$$

TABLEAU II

REPRODUCTIBILITÉ DU DOSAGE

(les résultats sont exprimés en μl d'EDTA 0.01 *M*)

	Moyenne	σ	*S.E.M.*	$m \pm 2$ *S.E.*	2 *S.E.* %
50 μl sol. calcium	16.39	0.20	0.07	16.39 ± 0.13	
Témoin	3.22	0.18	0.06	3.22 ± 0.12	
Différence	13.17		0.09	13.17 ± 0.18	1.4
100 μl sol. calcium	29.76	0.60	0.20	29.76 ± 0.40	
Témoin	3.53	0.17	0.05	3.53 ± 0.12	
Différence	26.23		0.21	26.23 ± 0.42	1.6

Ces résultats nous montrent que, compte-tenu du faible volume du prélèvement, la précision de la technique est excellente.

Nous avons vérifié que les autre métaux du sérum (plus spécialement le fer) n'interféraient pas. L'addition de cyanure, recommandée par BUCKLEY[14] nous est apparue inutile, les valeurs mesurées étant identiques en présence ou en l'absence de cyanure. Nous avons également constaté que le magnésium sérique, même à concentration 6 fois supérieure à la normale, n'introduisait pas de cause d'erreur dans le titrage du calcium (Tableau III).

TABLEAU III

INFLUENCE DU MAGNÉSIUM

(les résultats sont exprimés en mm)

Calcium μg/ml	*Magnésium* μg/ml	*mm*	*Calcium* μg/ml	*Magnésium* μg/ml	*mm*
0	24.5	0	60	30.4	91.5
0	121.5	0	80	0	120
40	0	60	80	30.4	121
40	30.4	59	100	0	150
60	0	90	100	30.4	150

Enfin, l'hémolyse n'influence pratiquement pas le dosage, comme le montrent les résultats du Tableau IV, obtenus en ajoutant à un sérum des volumes croissants d'un hémolysat centrifugé, préparé à partir de sang total.

TABLEAU IV

INFLUENCE DE L'HÉMOLYSE (LE DOSAGE A ÉTÉ EFFECTUÉ SUR 80 μl) DU MÉLANGE

(les résultats sont exprimés en mm)

Sérum ml	*Hémolysat* ml	*Eau* ml	*mm*
2	0.00	0.50	130
2	0.05	0.45	131.7
2	0.10	0.40	129
2	0.20	0.30	130

Bibliographie p. 363

REMERCIEMENTS

Ce travail a pu être réalisé grâce à une bourse d'Attaché de Recherche attribuée à l'un de nous (J. C.-M.) par l'Institut National d'Hygiène, que nous tenons à remercier.

RÉSUMÉ

La calcéine constitue un excellent indicateur dans le dosage complexométrique du calcium sérique: indicateur spécifique et stable. Par ailleurs, la disparition de sa fluorescence en l'absence d'ions Ca^{+2} libres est infiniment plus sensible que la modification de sa coloration. Nous proposons un microdosage de la calcémie fondé sur l'enregistrement automatique des variations de fluorescence de la calcéine, permettant d'apprécier la concentration en calcium sur un échantillon de sérum inférieur à 0.1 ml (de 0.02 à 0.1 ml) avec une précision de $\pm$ 1.5%.

SUMMARY

ULTRAMICRO DETERMINATION OF SERUM CALCIUM

Calcein is an excellent indicator for the complexometric determination of serum calcium, since it is specific and stable. The disappearance of its fluorescence in the absence of calcium ions is, however, much more sensitive than the colour change. A micromethod for determining calcium in cases of calcaemia is proposed, in which the variation of the fluorescence of calcein is automatically recorded. With this method it is possible to determine the calcium content in a sample of serum less than 0.1 ml (0.02–0.1 ml) with an accuracy of $\pm$ 1.5%.

BIBLIOGRAPHIE

1 B. KRAMER ET F. F. TISDALL, *J. Biol. Chem.*, 47 (1921) 475.
2 E. P. CLARK ET J. B. COLLIP, *J. Biol. Chem.*, 63 (1923) 461.
3 F. W. FALES, *J. Biol. Chem.*, 204 (1953) 577.
4 A. D. KENNY ET S. U. TOVERUD, *Anal. Chem.*, 26 (1954) 1059.
5 R. H. WILKINSON, *J. Clin. Pathol.*, 10 (1957) 126.
6 A. E. SOBEL ET A. HANOK, *Proc. Soc. Exptl. Biol. Med.*, 77 (1951) 737.
7 H. C. LEIFHEIT, *J. Lab. Clin. Med.*, 47 (1956) 623.
8 G. MATTIOLI, A. PIERANGELI ET C. DE RUBERTIS, *Boll. soc. ital. biol. sper.*, 33 (1957) 947.
9 J. CLEMENT-METRAL ET P. CARTIER, *Proc. 4th Intern. Congr. Biochemistry, Vienna*, 1958, 17–15, p. 200.
10 F. W. DIGGINS, *Analyst*, 80 (1955) 401.
11 T. T. GORSUCH ET A. M. POSNER, *Nature*, 176 (1955) 268.
12 D. MENDELSOHN, *S. African J. Med. Sci.*, 21 (1956) 57.
13 M. B. WILLIAM ET J. H. MOSER, *Anal. Chem.*, 25 (1953) 1414.
14 E. S. BUCKLEY, JR., J. G. GIBSON 2nd ET T. R. BORTOLOTTI, *J. Lab. Clin. Med.*, 38 (1951) 751.
15 A. C. KIBRICK, M. ROSS ET H. E. ROGERS, *Proc. Soc. Exptl. Biol. Med.*, 81 (1952) 353.
16 I. J. GREENBLATT ET S. HARTMAN, *Anal. Chem.*, 23 (1951) 1708.
17 H. DIEHL ET J. L. ELLINGBOE, *Anal. Chem.*, 28 (1956) 882.
18 B. M. TUCKER, *Analyst*, 82 (1957) 284.
19 D. N. BARON ET J. L. BELL, *Clin. Chim. Acta*, 2 (1957) 327.
20 J. KÖRBL ET F. VYDRA, *Chem. listy*, 51 (1957) 1457.
21 R. O. ASHBY ET M. ROBERTS, *J. Lab. Clin. Med.*, 49 (1957) 958.
22 I. M. BETT ET G. P. FRASER, *Biochem. J.*, 68 (1958) 13 P.
23 M. C. SANZ, *Clin. Chem.*, 3 (1957) 406.
24 M. A. ANDERSCH, *J. Lab. Clin. Med.*, 49 (1957) 486.

Reçu le 5 novembre 1958

THE ANTIPLASMIN EFFECT OF PLASMA FRACTIONS

M. RYBÁK AND J. REJNEK
Institute of Hematology and Blood Transfusion, Prague (Czechoslovakia)

The fibrinolytic system composed of proactivator, activator, plasminogen, plasmin and inhibitors[1] as constituents of the hemocoagulation system plays an important role in various bleeding states[2, 3], atherosclerosis[4], thrombophlebitis[5] and similar diseases. In normal states the plasmin level in the blood is sufficiently balanced by the presence of inhibitors (antiplasmin), the production of which in normal individuals is proportionate to the enzyme production. Every deviation from this equilibrium indicates a disturbance of some physiological function of the organism. Increased or lowered fibrinolytic activity can be found in a great number of disease states[6–8], after surgical operations[9, 10], ionizing irradiation[2], post-transfusion reactions[11], in leukemias[12] etc. The fibrinolytic activity represents the sum of the two main factors—plasmin and antiplasmin—and is regulated by hormones[13].

In order to determine these fibrinolytic factors, antiplasmin has to be separated from plasmin. This can be done either by dilution or fractionation. Plasmin is present in the euglobulin fraction[14], whereas the antifibrinolytic effect can be detected in the α-globulin[31] and albumin fraction[14]. We observed the nature and range of the inhibitory effect of the individual plasma fractions on the activity of plasmin. The following fractions were tested: fraction IV, V, IV+V, pasteurized fraction IV, V, IV+V, the α-globulin fraction from fraction III and IV and all other main fractions obtained by the separation of plasma with the help of preparative paper electrophoresis.

MATERIALS AND METHODS

Isolation of albumin from fractions IV + V

Albumin was isolated by using a modification of COHN's method 6 and 10[15]. The pasteurized preparation contained an average of 96% albumin, 3% α-globulin and 1% β-globulin. Fraction IV: 40% albumin, 25% α-globulin, 28% β-globulin, 7% γ-globulin.

Isolation of stable plasma solution from fraction IV + V

Isolation was carried out by the method mentioned for the isolation of albumin with the difference that fraction IV was not separated and was precipitated together with fraction V by 40% ethylalcohol at pH 4.8 and at a temperature of —8°. The precipitate was then extracted with 5% alcohol at pH 5.2 and lyophilized. A 5% solution was prepared and pasteurized for 10 h at 60°. Average composition of the fraction: 78% albumin, 9% α-globulin and 13% β-globulin.

Isolation of the α-globulins

The α- and β-globulin fraction which remains after the preparation of γ-globulin with the help of Zn^{+2} and Al^{+3} ions[16, 17], is separated after precipitation with Al^{+3} ions

References p 368

and washed with distilled water at pH 4.9 in order to remove the supernatant fluid containing γ-globulin from the precipitate. At this stage the precipitate contains a mixture of α- and β-globulins contaminated by a small amount of albumin. The precipitate is then dissolved in distilled water at pH 7.8 up to *ca.* 2% proteins, and the solution obtained is precipitated by adding Zn^{+2} ions up to the final concentration of 0.005 *M*; the albumin present is thus removed. The precipitate is then dissolved in a mixture of ion exchangers, a strongly acid katex and a strongly basic anex (1:2) in the H^+ and OH^- cycles. By adjusting the pH value from 7.1 to 5.8 the precipitation of the β-globulin and the lipoprotein fractions takes place. The supernatant fluid which contains only α-globulins is precipitated by adding Zn^{+2} ions up to a concentration of 0.005 *M*. The precipitate is then centrifuged and washed with distilled water by repeated suspension and centrifugation. The precipitate obtained by this method is dissolved in a 0.2% solution of natrium citrate, and by passing through a mixture of katex and anex in the H^+ and OH^- cycle it is freed from the remaining salts and dried by freeze sublimation. Average composition: 98–99% α-globulin, 1–2% albumin.

When using the ethanol fraction IV as starting material, the procedure is as follows: paste of fraction IV is suspended and dissolved in saline which precipitates after clearing and the addition of 0.005 M Zn^{+2} ions. The precipitated globulin fraction is dissolved after adjusting the pH to 5.6 and precipitated with the same volume of 0.005 *M* Al^{+3} ions in a complex with natrium tartrate pH 4.9. The precipitate thus obtained contains only α- and β-globulins with a small admixture of albumin and is dissolved by neutralisation. By adjusting the pH to 5.3, the resulting precipitate is removed and the supernatant now contains only pure α-globulin which can be precipitated by using ethanol or Zn^{+2}.

Preparative electrophoresis

Preparative electrophoresis according to LAURELL[18] from 2 ml serum yielded fractions α-, β- and γ-globulin and albumin. The protein concentration which differs in the various samples was adjusted by a veronal–citrate buffer of pH 8.6, *I* 0.1 to the same protein content (0.01%) and the purity determined by immunoelectrophoresis and paper electrophoresis. The influence of the fractions on fibrinolysis is evaluated in Table I.

Paper electrophoresis

Paper electrophoresis was carried out for 6 h in veronal–citrate buffer solution (pH 8.6, *I* 0.05) on Whatman paper No. 1 at 300 V. The electropherograms were stained with an acid solution of Bromphenol blue containing $HgCl_2$. The elution of the single zones for evaluation was carried out by a 1% solution of natrium carbonate in 50% methylalcohol and the amount of the eluted stains determined photometrically.

Immunoelectrophoresis

Immunoelectrophoretic analysis was carried out in a micromodification by an adaptation of SCHEIDEGGER's method[19]. The precipitating antihuman horse serum used was supplied by the Pasteur Institute in Paris.

Protein determination

The proteins were determined by the Kjeldahl method, a modification according to MARKHAM[20], and the biuret reaction[21].

References p. 368

The determination of fibrinolysis and its inhibitors

Determination of plasmin. A modification of FERGUSON's test-tube method[23, 24] was used for the determination of the activity of the plasmin solution[22]. The end-point of the fibrinolysis is indicated by the rising of air bubbles to the surface.

Determination of the inhibitory effect. The inhibitory effect of the single fractions was determined by the prolongation of the time of lysis of a fibrin coagulum by a standard plasmin solution at 37°. This coagulum was obtained by the coagulation of a 0.4% standard beef fibrinogen solution[27]. The inhibition was expressed quantitatively in inhibitory units. One inhibitory unit is able to neutralize one unit of plasmin activity. The degree to which changes of activity depend on the fraction with antiplasmin effect is expressed in Figs. 1, 2 and 3.

RESULTS AND DISCUSSION

By comparing the inhibitory effect of the single fractions (Fig. 1) separated by preparative electrophoresis, the maximum inhibitory effect was found in the region of the α_1 and α_2-globulins, and a slightly smaller but still marked effect in the albumin fraction. The presence of plasminogen in the β-globulin fraction caused an increase of fibrinolytic activity in the presence of streptokinase.

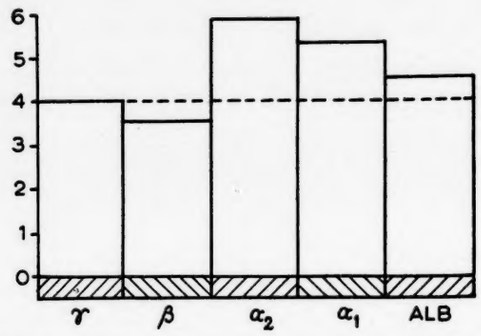


Fig. 1. The influence of fractions isolated by preparative electrophoresis on the activity of plasmin. Time of lysis of a standard fibrin coagulum after adding 1 ml 0.01% solution of the fraction.

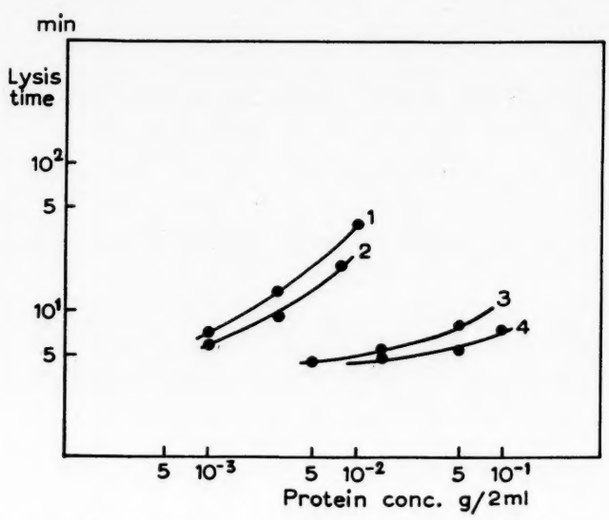


Fig. 2. Time of lysis of standard fibrin coagulum depending on the concentration of fraction IV, IV + V and α-globulin; 1 = α-globulin, 2 = fr. IV, 3 = fr. IV + V, 4 = fr. V.

References p. 368

The inhibitory effect of the fractions containing α-globulins prepared by cold ethanol fractionation commonly used for therapeutic purposes fraction V and IV+V, fraction IV, and of the α-globulins from fraction III and IV was observed. The antiplasmin activity of the various fractions IV+V increased according to the α-globulin content which varied between 7–12%. The antiplasmin effect of albumin observed by MACFARLANE and confirmed by our results can be attributed to a certain amount of α_1-globulins in the purified albumin preparations (due to the proximity of their isoelectric points) (Fig. 2).

The influence of pasteurization and stabilization on the change of the inhibitory ability of the fractions was studied. If fractions V and IV+V were heated without adding the stabilizer, the inhibitory ability increased more rapidly than in the presence of the stabilizer (acetyl-*dl*-tryptophan, pH 7.0). The inhibitory activity of the α-globulins and fraction IV decreases sharply at the beginning of heating, starts in-

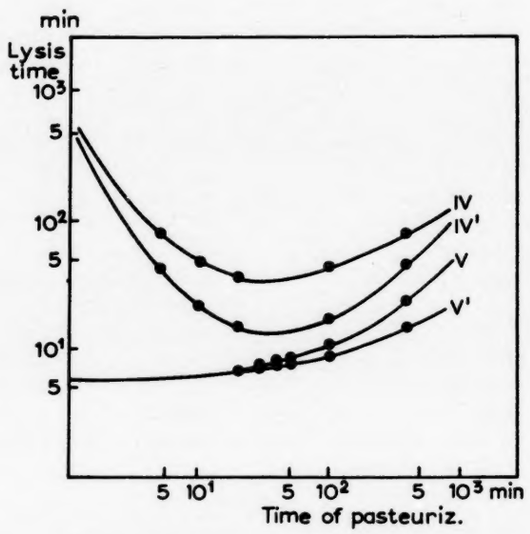


Fig. 3. Influence of pasteurization and stabilisation (0.04 *M* acetyl-*dl*-tryptophan) on the inhibitory ability of fractions IV and V (1% solutions). The result represents the average of 5 determinations; IV, V: non-stabilized fraction; IV′, V′: stabilized fraction.

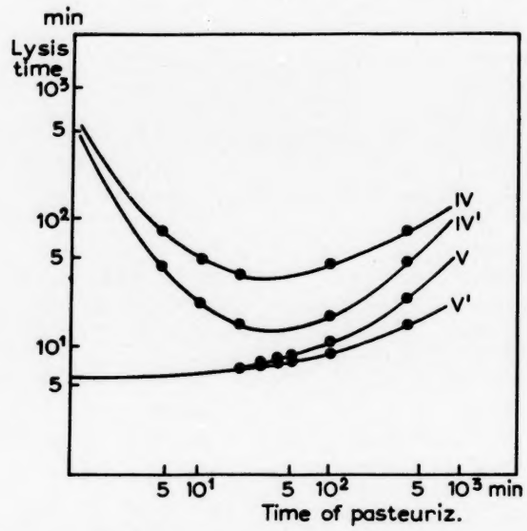
creasing after 1 h and reaches a level incomparably higher than that of albumin and fraction IV+V after 10 h heating. After heating for 5 min at 80–90°, the inhibitory effect of all fractions increased manifold (the time of lysis of the fibrin coagulum lasting several hours). This indicates that this kind of inhibition is caused by partial denaturation of the protein chain which renders the splitting by plasmin possible[25]. Pasteurization thus gives rise to a new proteolytic substrate which, by binding part of the plasmin activity, competes with fibrinolysis whilst the original activity is destroyed by heating (Fig. 3).

FIRKIN and others[2, 26] used albumin with success in the therapy of hemorrhagic diathesis caused by a circulating fibrinolysin. On the basis of the *in vitro* results shown in Figs. 2 and 3, a considerable antiplasmin effect of fraction IV+V, IV and purified α-globulin could be expected *in vivo*. This effect is caused by counteracting the action of increased fibrinolytic activity. In cases of fibrinogen deficiency in the blood circulation (afibrinogenemia) through increased fibrinolytic activity, these fractions could

References p. 368

nhibitory effect of the fractions containing α-globulins prepared by cold ıctionation commonly used for therapeutic purposes fraction V and IV+V, V, and of the α-globulins from fraction III and IV was observed. The anti-ctivity of the various fractions IV+V increased according to the α-globulin hich varied between 7–12%. The antiplasmin effect of albumin observed by ANE and confirmed by our results can be attributed to a certain amount of ıs in the purified albumin preparations (due to the proximity of their iso-ints) (Fig. 2).

ıfluence of pasteurization and stabilization on the change of the inhibitory the fractions was studied. If fractions V and IV+V were heated without e stabilizer, the inhibitory ability increased more rapidly than in the f the stabilizer (acetyl-*dl*-tryptophan, pH 7.0). The inhibitory activity of the s and fraction IV decreases sharply at the beginning of heating, starts in-



ıence of pasteurization and stabilisation (0.04 *M* acetyl-*dl*-tryptophan) on the inhibi-of fractions IV and V (1% solutions). The result represents the average of 5 deter-minations; IV, V: non-stabilized fraction; IV′, V′: stabilized fraction.

fter 1 h and reaches a level incomparably higher than that of albumin and V+V after 10 h heating. After heating for 5 min at 80–90°, the inhibitory l fractions increased manifold(the time of lysis of the fibrin coagulum lasting urs). This indicates that this kind of inhibition is caused by partial dena-f the protein chain which renders the splitting by plasmin possible[25]. Pas-n thus gives rise to a new proteolytic substrate which, by binding part of in activity, competes with fibrinolysis whilst the original activity is de-y heating (Fig. 3).

IN and others[2, 26] used albumin with success in the therapy of hemorrhagic caused by a circulating fibrinolysin. On the basis of the *in vitro* results shown and 3, a considerable antiplasmin effect of fraction IV+V, IV and purified could be expected *in vivo*. This effect is caused by counteracting the action ed fibrinolytic activity. In cases of fibrinogen deficiency in the blood cir-ıfibrinogenemia) through increased fibrinolytic activity, these fractions could

prevent the rapid lysis of the applied fibrinogen and thus heighten its effect which in these cases proves of little value[28].

SUMMARY

The antiplasmin activity of plasma fractions isolated by preparative electrophoresis, ethanol fractionation and fractionation by metal salts was studied. This activity is confined to the fractions containing α-globulins. By denaturation during pasteurization of these fractions, a new substrate for plasmin is obtained which binds part of the plasmin activity and inhibits fibrinolysis. The original inhibitory activity is for the greater part destroyed by heating. From the results obtained, a considerable antiplasmin effect of the fractions with higher content of α-globulin could be expected *in vivo*.

REFERENCES

1 S. MÜLLERTZ, *Acta Physiol. Scand.*, 38, Suppl. 130 (1956).
2 B. G. FIRKIN, S. H. REED AND C. R. B. BLACKBURN, *Brit. J. Heamatol.*, 3 (1957) 193.
3 F. X. PALETTA *Plastic and Reconstr. Surg.*, 19 (1957) 1.
4 J. R. O'BRIEN, *Am. J. Med. Sci.*, 234 (1957) 4.
5 T. ASTRUP, *Lancet*, 251 (1956) 11.
6 R. MACFARLANE AND R. BRIGGS, *Blood*, 3 (1948) 1167.
7 M. STEFANINI, *Blood*, 7 (1952) 1044.
8 E. W. GOODPASTURE, *Johns Hopkins Hosp. Bull.*, 25 (1914) 330.
9 L. J. LOMBARDO, *J. Urol.* 77 (1957) 2.
10 O. D. RATNOFF, *J. Clin. Invest.*, 31 (1952) 521.
11 J. PANASEWISZ AND W. NIEWIAROWSKI, *Polskie Arch. Med. Wewnet.* 26 (1956) 1.
12 A. A. COOPERGBERG AND G. M. NEIMAN, *Ann. Internal Med.*, 42 (1955) 706.
13 G. UNGAR AND E. DAMGAARD, *J. Exptl. Med.*, 93 (1951) 89.
14 R. MACFARLAN AND J. PILLING, *Lancet*, 2 (1946) 562.
15 M. RYBÁK, unpublished results.
16 J. REJNEK AND F. ŠKVAŘIL, *Coll. Czech. Chem. Communs.*, 22 (1957) 1489.
17 J. REJNEK AND F. ŠKVAŘIL, *Coll. Czech. Chem. Communs.*, 23 (1958) 773.
18 A. B. LAURELL, *Acta Pathol. Microbiol. Scand.*, suppl. 103, (1955).
19 J. J. SCHEIDEGGER *Intern. Arch. Allergy*, 7 (1955) 103.
20 R. MARKHAM, *Biochem. J.*, 36 (1942) 790.
21 J. HOŘEJŠÍ, *Bílkoviny krevní plasmy*, Zdr. nakl. Praha, 1956.
22 M. RYBÁK, *Clin Chim. Acta*, in the press.
23 J. H. LEWIS AND J. FERGUSON, *Am. J. Physiol.*, 170 (1952) 636.
24 J. B. FISHMAN AND D. L. KLINE, *Proc. Soc. Exptl. Biol. Med.*, 91 (1956) 323.
25 T. GODFRAIND, *Arch. intern. pharmacodynamic* 105, 3/4 (1956).
26 E. V. Z. SCOTT, W. F. M. MATTHEWS, C. E. BUTTERWORTH, JR., AND W. M. FROMMEYER, *Surg. Gynecol. Obstet.*, 99 (1954) 679.
27 R. A. KEKWICK, *Biochem. J.*, 60 (1955) 671.
28 J. DUCOS AND R. BIERMÉ, *Sang*, 28 (1957) 7.

Received September 11th, 1958

STUDIES ON FAST-MOVING ALBUMINS IN HUMAN SERUM*

S. A. KARJALA AND Y. NAKAYAMA

The University of Texas, M. D. Anderson Hospital and Tumor Institute, Department of Biology, Houston, Texas (U.S.A.)

INTRODUCTION

The presence of two electrophoretic components in normal human and animal albumin has been reported by numerous investigators[1-8]. All these studies relate to the formation in acid solution of modified products of an albumin which was initially electrophoretically homogeneous at pH values near neutrality, or to transition of part of the albumin to a faster migrating form.

There have been, however, numerous reports in the literature on the presence of a fast-moving protein in human serum since the initial demonstration by KABAT, MOORE AND LANDOW[9] of the presence of a fast-moving protein in cerebrospinal fluid. A fast-moving albumin was demonstrated to be present in human serum by WILLIAMS AND GRABAR[10] by immuno-electrophoresis, and SCHULTZE, SCHÖNENBERGER AND SCHWICK[11], in a paper which also includes a comprehensive review of the earlier work, described the isolation of a prealbumin by a combination of precipitation and electrophoretic methods.

This prealbumin had an immunological specificity different from that of albumin. Immunoelectrophoresis by the procedure of GRABAR AND WILLIAMS[12] developed a strong precipitin arc of rabbit anti-prealbumin serum with prealbumin, but gave only a weak arc with albumin. A strong arc of identity was found between prealbumin, human serum and the antiserum to prealbumin, indicating the presence in human serum of a component with the specificity of prealbumin, and showing that it is not an artefact arising from the somewhat drastic isolation procedure.

LARSON AND FEINBERG[13], using continuous paper electrophoresis, separated albumin, isolated from normal human serum (NHS) by ethanol fractionation, into numerous fractions, and reported as many as six different components in some lots of albumin, using the Oudin gel-diffusion technique. Since the unfractionated albumin was used for preparation of the antiserum, the Oudin method alone will not differentiate between separate antigen–antibody systems and proteins of different diffusion rates through agar gel but with similar albumin specificities.

BAUDOUIN, LEWIN AMD HILLION[14] showed the presence of fast-moving albumins by paper electrophoresis, and DUMAZERT, GHIGLIONE AND BOZZI-TICHADOU[15] reported the separation of human and bovine serum albumin into at least six components by electrophoresis on columns of cellulose powder.

JIRGENSONS[16] has shown that significant differences exist between various specimens of serum albumin in optical rotation and viscosity. An exhaustive study of the possible errors and impurities involved led to the conclusion that these differences are due to the existence of different albumins.

* Supported in part by a grant from the National Institutes of Health, U.S.P.H.S., Grant No. C-3416

References p. 373

In the work to be described below, it has been found that human serum albumin in serum from outdated blood-bank supplies can be fractionated by continuous paper electrophoresis into numerous fractions of increasing electrophoretic mobility, but in all cases the immunological specificity is that of crystalline human serum albumin. The gradual increase in mobility from one fraction to the next suggests the presence of a multiplicity of components, with no single predominant fast-moving component.

MATERIAL AND METHODS

A total of 300 ml of serum from outdated blood obtained from the blood bank was fractionated in portions in the Durrum-type Beckman-Spinco continuous paper electrophoresis apparatus. Each of the fractions was checked qualitatively for the presence of protein by testing aliquots by the Folin method as modified by LOWRY *et al.*[17]. The top five fractions of each fractionation were combined and refractionated at a current density of 35 mA., 500 V, and a flow of 1 ml/h in 0.02 ionic strength veronal buffer of pH 8.6.

Paper electrophoresis runs were made in the Beckman-Spinco paper electrophoresis apparatus in 0.05 *ionic strenght* veronal buffer at 4.5 mA, per cell for 16 h, after which the strips were stained with bromphenol blue.

The agar gel electrophoresis and immunoelectrophoresis runs were made in a modification of the apparatus of GRABAR AND WILLIAMS[12]. Details on the modification, method of purification of the gel, staining, and other points are described in an earlier paper[18].

Agar gel columns were prepared by coating the inside of a glass tube having a removable sintered glass plate with hot 3% agar, drying the tube in an oven, replacing the sintered glass plate, and layering it with 1% agar which had been cooled sufficiently to be whipped into a slurry with a glass rod. The slurry was carefully covered, and the tubes filled, with a hot 1% agar solution to the desired height and allowed to gel. A layer of serum or other sample was placed at the top of the column, treated with an equal volume of warm 1% agar, and this layer covered with warm 1% agar after it has solidified. The agar was dissolved in 0.9% saline containing 1/10,000 methiolate, and the same solvent was used for eluting the protein.

Rabbits were immunized against NHS and against crystalline human serum albumin (CHSA) by the usual procedure of 4 injections per week for 4 weeks, with increasing antigen dosages each week, after which the animals were rested for 5 to 7 days and bled by heart puncture.

DISCUSSION AND RESULTS

When NHS was placed on an agar gel column and the protein mixture was eluted by gravity with 0.9% saline, fractions of approximately 0.5 ml per day were obtained on 10 × 300 mm columns, using a column height of 200 mm. No protein could be detected in aliquots of the eluates by the Folin reagent for 10–14 days. The first fraction to show the presence of protein was dialyzed against distilled water, lyophilized, dissolved in a drop of saline, and subjected to agar gel electrophoresis against a sample of CHSA. No spot could be developed for the eluted protein on staining the agar plate with azocarmine, but when the agar plate after electrophoresis was set up

References p. 373

for gel diffusion against an anti-CHSA antibody solution concentrated by the procedure of NICHOL AND DEUTSCH[19], a distinct arc of precipitation of a fast-moving albumin was observed, as shown in Fig. 1. The position of the two main precipitin arcs clearly shows the increased electrophoretic mobility of the fast-moving albumin. A trace of α-globulin in the CHSA is also shown by a faint precipitin arc immediately behind the albumin arc for this system. The presence of α-globulin as an impurity in purified serum albumin has already been shown by immunoelectrophoresis by SCHULTZ *et al.*[11] and by CAMPBELL AND STONE[20].

Attempts to isolate a fast-moving protein from solutions of CHSA on agar gel columns were not successful. In all cases, the first portion of eluate to give a positive Folin reaction showed a protein spot on agar gel electrophoresis which had the same

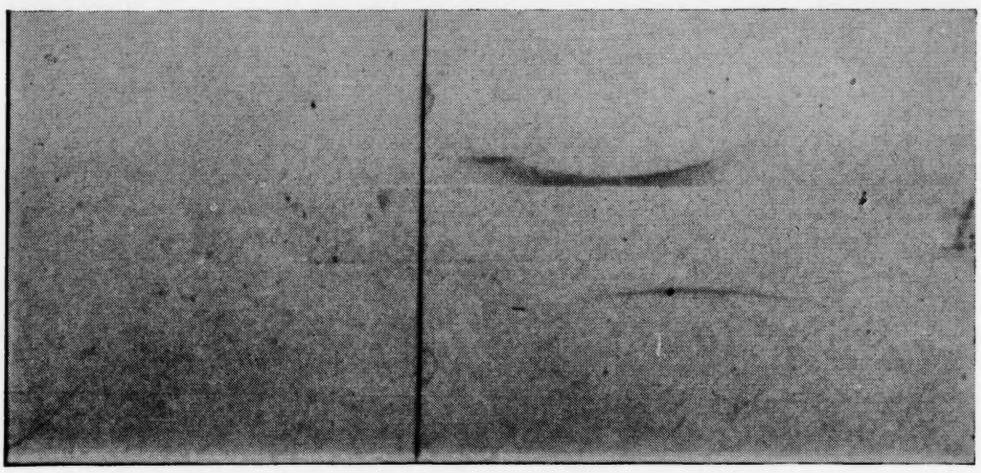


Fig. 1. Immunoelectrophoresis of agar gel column eluate (bottom) and CHSA (top) against a concentrated anti-CHSA antiserum. The faint precipitin arc for an α-globulin impurity in the CHSA lies immediately behind the albumin arc.

electrophoretic mobility as that of the initial crystalline protein. This suggests that the fast-moving albumin components may be of smaller molecular size and higher solubility, and are fractionated out during the crystallization of the albumin. It was noted that on agar gel and paper strip electrophoresis the spot for albumin in NHS has a slightly greater mobility than did the CHSA, as though the "light" components of the whole albumin contribute sufficient increase in electrophoretic mobility to produce a distinctly faster mobility than that of the "heavy" crystalline protein from which the lighter components have been removed.

Since the amounts of eluate and protein yields from agar gel columns were very low as a result of the slow elution rate, studies were made on the fractionation of the proteins by continuous paper electrophoresis. The albumin fractions of numerous NHS fractionations were combined and separated again by continuous paper electrophoresis, the fractions from drip points 25 through 29 being saved. Fig. 2 shows the paper strip electrophoresis results on these fractions, together with control strips of CHSA and of NHS. A slight increase in mobility is observable from fraction 25 through 29 over that of the albumin NHS, which, in turn, shows a slightly greater mobility than does CHSA. The amount of material in fraction 29 was so small that the protein was absorbed on the paper before maximum mobility was attained. The immuno-

References p. 373

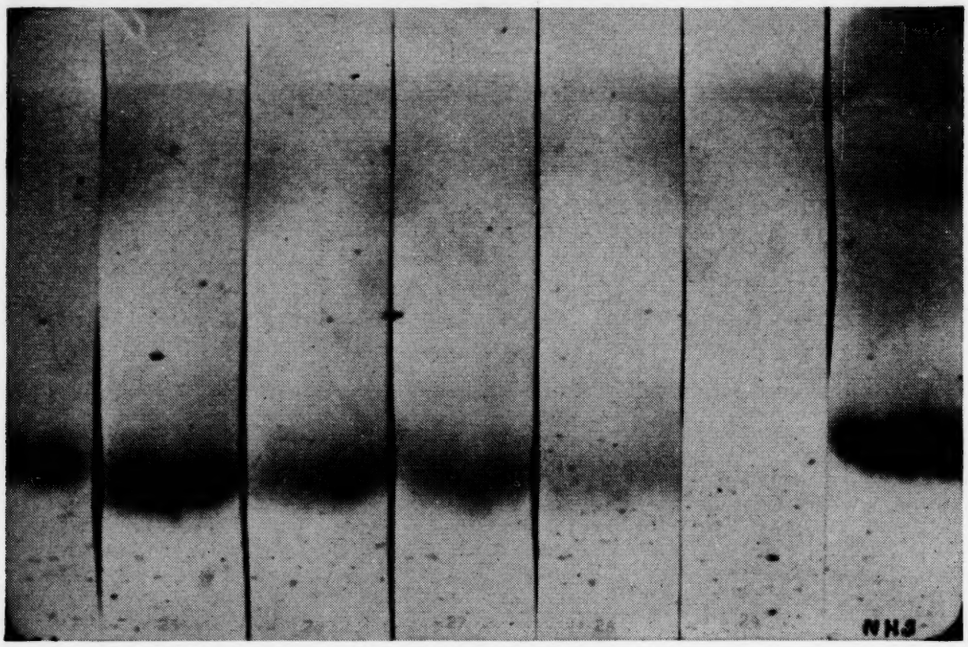


Fig. 2. Paper strip electrophoresis of fractions 25 through 29 of NHS albumin, together with strips of NHS (right) and CHSA (left).

electrophoresis runs, however, demonstrate the presence of fast-moving albumin in this fraction. Adsorption is also a factor in paper strip electrophoresis, and differences in mobility are not as apparent as in the agar gel electrophoresis runs.

In Fig. 3 are shown the results obtained after immunoelectrophoresis of albumin fractions 25, 27 and 28 run with samples of CHSA, and set up against a rabbit anti-

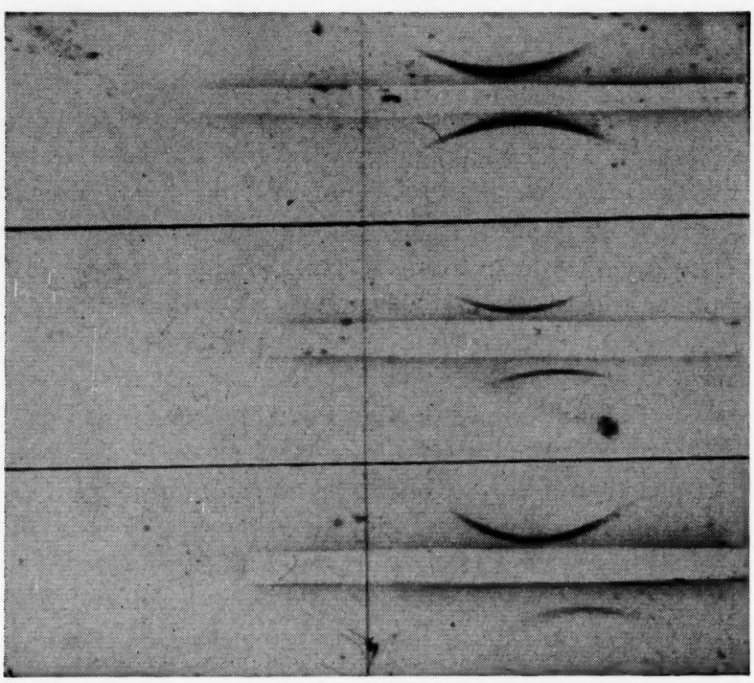


Fig. 3. Immunoelectrophoresis of fast-moving albumin fractions 25 (upper), 27 (middle), and 28 (lower plate). Each fraction was run with CHSA against an anti-CHSA antiserum, the arc for the fast-moving fraction being the lower one on each plate.

References p. 373

serum to CHSA, demonstrating that the fractions all react with this antiserum. Since the antiserum had been prepared using CHSA as an antigen, and the CHSA had not shown the presence of fast-moving fractions upon elution from agar gel columns, it was concluded that there is no difference in immunological specificity between CHSA and the fast-moving fractions.

By the use of continuous paper electrophoresis or by elution from agar gel columns it has been demonstrated that fractions can be obtained with electrophoretic mobilities greater than that of CHSA, but in no case was evidence of a fast-moving protein found that had an immunological specificity different from that of CHSA.

All runs were made on pooled samples of serum from outdated blood. A single sample of fresh human serum on continuous paper electrophoresis gave a spread of albumin over seven drip points but none of these fractions showed appreciable amounts of fast-moving material.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Mr. E. HORNBERGER, and the advice and comments of Dr. B. JIRGENSONS. We wish to thank Dr. F. F. JOHNSON of Cutter laboratories for furnishing a generous supply of crystalline human serum albumin for use in this investigation.

SUMMARY

Normal human serum can be separated by continuous paper electrophoresis or by elution from agar gel columns to yield fractions with electrophoretic mobilities greater than that of crystalline human serum albumin, but in no case was evidence of a fast-moving protein found that had an immunological specificity different from that of crystalline human serum albumin. The prealbumin found by the German workers does not appear to be an albumin, but may be a fast-moving globulin such as a glycoprotein.

REFERENCES

1 L. F. HEWITT, *Biochem. J.*, 30 (1936) 2229; 31 (1937) 360.
2 R. A. KEKWICK, *Biochem. J.* 32 (1938) 552.
3 T. L. MCMEEKIN, *J. Am. Chem. Soc.*, 61 (1939) 2884.
4 D. G. SHARP, G. R. COOPER, J. O. ERICKSON, AND H. NEURATH, *J. Biol. Chem.*, 144 (1942) 139.
5 J. A. LUETSCHER, Jr., *J. Am. Chem. Soc.*, 61 (1939) 2888.
6 P. A. CHARLWOOD, *Biochem. J.*, 52 (1952) 279; 56 (1954) 259.
7 A. KELTZ AND J. W. MEHL, *J. Am. Chem. Soc.*, 77 (1955) 5764.
8 L. AOKI AND J. F. FOSTER, *J. Am. Chem. Soc.*, 78 (1956) 3538; 79 (1957) 3385.
9 E. A. KABAT, D. H. MOORE, AND H. LANDOW, *J. Clin. Invest.*, 21 (1942) 571.
10 C. A. WILLIAMS, JR. AND P. GRABAR, *J. Immunol.*, 74 (1955) 158.
11 H. E. SCHULTZE, M. SCHÖNENBERGER, AND G. SCHWICK, *Biochem. Z.*, 328 (1956) 267
12 P. GRABAR AND C. A. WILLIAMS, JR., *Biochim. Biophys. Acta*, 17 (1955) 67.
13 D. L. LARSON AND R. FEINBERG, *Science*, 120 (1954) 426.
14 A. BAUDOUIN, J. LEWIN, AND P. HILLION, *Compt. rend. soc. biol.*, 150 (1956) 892.
15 C. DUMAZERT, C. GHIGLIONE, AND M. BOZZI-TICHADOU, *Bull. soc. chim. biol.*, 37 (1955) 123.
16 B. JIRGENSONS, *Makromol. Chem.*, 21 (1956) 179.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
18 S. A. KARJALA, *Makromol. Chem.*, in the press.
19 J. C. NICHOL AND H. F. DEUTSCH, *J. Am. Chem. Soc.*, 70 (1948) 80.
20 P. N. CAMPBELL AND N. E. STONE, *Biochem. J.*, 66 (1957) 19.

Received August 4th, 1958

SERIAL ELECTROPHORETIC STUDIES OF THE SERUM GLYCOPROTEINS AND PROTEINS IN PATIENTS WITH PULMONARY TUBERCULOSIS*

S. H. LAWRENCE, H. E. WEIMER, AND D. SALKIN

Biochemistry Research Laboratory, Veterans Administration Hospital, San Fernando, and Departments of Infectious Diseases and Medicine, School of Medicine, University of California, Los Angeles, Calif. (U.S.A.)

The demonstration of significant changes in the concentration and distribution of the carbohydrate-containing proteins of serum in various pathologic states has led to renewed clinical interest in this class of conjugated proteins. The development of adequate staining procedures for the protein-bound carbohydrates of serum following separation by filter paper electrophoresis[1, 2] has provided a new approach for their investigation. In previous reports from this laboratory[3, 4], alterations in serum glycoprotein and protein patterns in pulmonary tuberculosis had been studied by chemical separation methods. Filter paper electrophoresis seemed to offer certain advantages over the precipitation techniques with respect to simplicity and economies in time, equipment and serum.

In the present study, serum glycoprotein and protein patterns have been determined by zone electrophoresis in 30 normal individuals and in 101 consecutive tuberculosis admissions to the hospital. Serial studies were carried out on 16 patients who were the most acutely ill and whose serum exhibited the most marked deviations from normal electrophoretic patterns upon admission.

MATERIALS AND METHODS

Electrophoresis. Electrophoresis was carried out in a Durrum type apparatus. Schleicher and Schüll 2043 A filter paper was used for protein and Whatman No. 2 for glycoprotein determinations with serum samples of 0.006 ml and 0.05 ml, respectively. The strips were run in parallel for 16 h with a current of 3 mA veronal buffer, pH 8.6, ionic strength 0.075 was used. Strips were stained for protein with bromphenol blue[5] and for glycoprotein with the periodic acid–Schiff reagents[2]. The relative amounts of glycoprotein and protein in the serum fractions were determined by an integrating automatic scanner (Analytrol**). The glycoprotein curves were marked by superimposition with special reference to the α_2-globulin peaks. Absolute concentrations were estimated by reference to total serum glycoprotein and total serum protein concentrations[3]. The mean, standard error of the mean t, and probability values were computed by standard methods.[6]

Clinical appraisal. Inasmuch as it was previously shown[4] that serum glycoprotein elevations in pulmonary tuberculosis were more closely related to the amount of

* Supported in part by a grant from the Tuberculosis and Health Association of Los Angeles County.

** Beckman R/Spinco Division, Palo Alto, California, U.S.A.

References p. 377

exudative disease than to the extent of disease as estimated by the National Tuberculosis Association classification[7], the following categories were employed:

Class I Minimal amount of exudative disease or none at all (a small amount of fibrotic disease might be present).

Class II Exudative disease present to the extent of a moderately advanced lesion. Additional fibrotic or productive disease to minimal or moderate extent did not modify the classification.

Class III Extensive exudation throughout an entire lobe. Fibrotic disease throughout all lobes was indicated in this category.

Class IV Exudation throughout the equivalent of one entire lung.

Class V Exudation throughout all lung fields.

RESULTS AND DISCUSSION

The results of the serum glycoprotein determinations are summarized in Table I. Significant elevations in total glycoprotein concentrations occurred in patients with extensive, exudative disease due primarily to the increase in the bound-carbohydrates of the α_2- and β-globulin fractions. In Class III patients, statistically significant increases were found in all serum fractions with the exception of the α_1-globulin. Only in the most acutely ill patients did significant elevations occur in the α_1-globulin fraction. This observation contrasts with the results of HIRSCH AND CATTANEO[8] who found increases in the α_1-protein-bound carbohydrates in all patients with active disease, irrespective of classification.

TABLE I

THE EFFECTS OF PULMONARY TUBERCULOSIS ON THE CONCENTRATION AND DISTRIBUTION OF THE PROTEIN-BOUND CARBOHYDRATES OF SERUM[a]

Group	*No. of cases*	*Total serum*[b] *glycoprotein mg/100 ml*	*Albumin*[b] *glycoprotein mg/100 ml*	*Globulin glycoproteins*			
				α_1[b] *mg/100 ml*	α_2[b] *mg/100 ml*	β[b] *mg/100 ml*	γ[b] *mg/100 ml*
Normal	30	110 ± 6.1	16 ± 3.1	22 ± 2.0	26 ± 3.8	26 ± 2.5	20 ± 1.5
Pulmonary tuberculosis							
Class I	20	110 ± 6.2	19 ± 2.2	18 ± 2.2	27 ± 2.4	26 ± 2.3	20 ± 4.1
Class II	32	125 ± 5.1*	21 ± 3.5	19 ± 2.4	32 ± 4.1	31 ± 3.5	22 ± 3.2
Class III	21	170 ± 8.0**	29 ± 3.5**	27 ± 7.0	49 ± 3.4**	34 ± 2.5*	31 ± 1.5**
Class IV	12	171 ± 9.1**	22 ± 4.1	27 ± 3.4	60 ± 7.1**	34 ± 2.2*	28 ± 3.3*
Class V	16	201 ± 4.5**	19 ± 2.5	37 ± 3.4**	73 ± 7.8**	45 ± 4.9**	27 ± 5.1
Class V[c]	16	111 ± 2.7	15 ± 1.5	23 ± 1.3	25 ± 1.1	27 ± 1.3	21 ± 1.5

[a] The use of the term glycoprotein with respect to the serum fractions is not intended to imply that only a single carbohydrate-containing protein is involved.

[b] Including the standard error of the mean; statistically significant differences from normal values are indicated: * $P = <0.05 >0.01$; ** $P = <0.01$.

[c] Following 3 months successful therapy (streptomycin, isoniazid, and *p*-aminosalicylic acid).

Changes in the serum protein patterns as determined by zone electrophoresis (Table II) were similar to those reported by SEIBERT *et al.*[9] and by BALDWIN AND

References p. 377

ILAND [10] who employed a Tiselius apparatus. In patients with extensive disease, there is a decrease in serum albumin concomitant with increases in the α_2- and γ-globulin fractions. The γ-globulin increase occurred with less disease and may be related to immunologic mechanisms [9].

TABLE II

THE EFFECTS OF PULMONARY TUBERCULOSIS ON THE CONCENTRATION AND DISTRIBUTION OF THE SERUM PROTEINS

Group	*No. of cases*	*Total serum[a] protein g/100 ml*	*Albumin[a] g/100 ml*	*Globulins*			
				α_1[a] *g/100 ml*	α_2[a] *g/100 ml*	β[a] *g/100 ml*	γ[a] *g/100 ml*
Normal	30	7.1 ± 0.20	4.9 ± 0.21	0.2 ± 0.04	0.4 ± 0.06	0.6 ± 0.04	1.0 ± 0.12
Pulmonary tuberculosis							
Class I	20	6.9 ± 0.19	4.6 ± 0.18	0.2 ± 0.02	0.5 ± 0.03	0.6 ± 0.03	1.0 ± 0.13
Class II	32	7.0 ± 0.21	4.7 ± 0.18	0.2 ± 0.02	0.5 ± 0.01	0.7 ± 0.03	0.9 ± 0.12
Class III	21	7.1 ± 0.22	4.2 ± 0.20*	0.3 ± 0.03	0.5 ± 0.04	0.7 ± 0.04	1.4 ± 0.20
Class IV	12	6.8 ± 0.20	3.7 ± 0.25**	0.3 ± 0.06	0.6 ± 0.08*	0.7 ± 0.04	1.5 ± 0.20*
Class V	16	6.4 ± 0.20*	3.2 ± 0.20**	0.3 ± 0.09	0.7 ± 0.08**	0.7 ± 0.07	1.5 ± 0.10**
Class V[b]	16	7.2 ± 0.12	4.9 ± 0.12	0.2 ± 0.01	0.4 ± 0.01	0.6 ± 0.01	1.1 ± 0.03

[a] Including the standard error of the mean; statistically significant differences from normal values are indicated: * $P = <0.05 >0.01$; ** $P = <0.01$.

[b] Following 3 months successful therapy (streptomycin, isoniazid, and *p*-aminosalicylic acid).

In an attempt to further define the influence of pulmonary tuberculosis on the glycoproteins and proteins of serum, a study was made of their relationships (Table III). Increased ratios were observed for whole serum and the albumin, α_2- and β-globulin fractions in patients with extensive disease. It should be noted, however, that

TABLE III

THE EFFECTS OF PULMONARY TUBERCULOSIS ON SERUM GLYCOPROTEIN–PROTEIN RELATIONSHIPS

	Total serum[a] glycoprotein ÷ total serum protein × 100	*Albumin[a] glycoprotein ÷ albumin protein × 100*	*Globulin glycoprotein ÷ globulin protein × 100*			
			α_1[a]	α_2[a]	β[a]	γ[a]
	%	%	%	%	%	%
Normal	1.6 ± 0.8	0.3 ± 0.03	11.0 ± 1.42	6.5 ± 0.40	4.3 ± 0.38	2.0 ± 0.23
Pulmonary tuberculosis						
Class I	1.6 ± 0.07	0.4 ± 0.04	9.0 ± 1.90	5.4 ± 1.19	4.3 ± 0.43	2.0 ± 0.50
Class II	1.8 ± 0.08	0.4 ± 0.04	9.5 ± 1.31	6.4 ± 0.38	4.4 ± 0.36	2.4 ± 0.25
Class III	2.4 ± 0.14**	0.7 ± 0.07**	9.0 ± 1.79	9.8 ± 1.07	4.9 ± 0.55	2.2 ± 0.18
Class IV	2.5 ± 0.28**	0.6 ± 0.12**	9.0 ± 2.30	10.0 ± 0.82**	4.9 ± 0.72	1.9 ± 0.14
Class V	3.1 ± 0.20**	0.6 ± 0.26	12.3 ± 2.66	10.4 ± 1.49*	6.4 ± 0.60*	1.8 ± 0.20
Class V[b]	1.5 ± 0.05	0.3 ± 0.03	11.5 ± 0.74	6.3 ± 0.34	4.5 ± 0.22	1.9 ± 0.16

[a] Including the standard error of the mean; significant differences from normal values are indicated: * $P = <0.05 >0.01$; ** $P = <0.01$.

[b] Following 3 months successful therapy (streptomycin, isoniazid, and *p*-aminosalicylic acid).

References p. 377

considerable variability occurred as indicated by the magnitude of the standard error of the mean and the results were not statistically significant in all cases.

The rapid return to normal of the serum glycoprotein and protein patterns in patients who responded to therapy was demonstrated by the values obtained for the Class V patients after 3 months treatment (Tables I, II, III).

For comparison with results obtained in this laboratory by other chemical fractionation procedures[3, 4], the patients were also categorized by the National Tuberculosis Association classification[7] with respect to extent of disease and by the author's estimation of the character of the disease (exudative, productive, fibrotic) from chest roentgenograms. When the patients were grouped into these classifications and their serum values compared, the results were in good agreement with those previously described[3, 4, 8–10]. Patients with far advanced disease exhibited greater alterations in their serum glycoprotein and protein patterns than those with minimal or moderate disease, and more pronounced changes occurred in the exudative group than in the productive or fibrotic groups.

ACKNOWLEDGEMENT

The technical assistance of Mrs. ELLEN AIKEN is gratefully acknowledged.

SUMMARY

The effects of pulmonary tuberculosis on the concentration and distribution of the protein-bound carbohydrates and proteins of serum have been investigated by filter paper electrophoresis in 101 patients and 30 normal individuals. Statistically significant increases in total serum glycoprotein and the protein-bound carbohydrates of the α_2- and β-globulin fractions occurred in patients with extensive, exudative disease concomitant with increases in the protein components of the α_2- and γ-globulin fractions and with a decrease in serum albumin. The serum glycoprotein and protein patterns in acutely ill patients who responded to chemotherapy rapidly returned to normal.

REFERENCES

1 E. KOIW AND A. GRONWALL, *Scand. J. Clin. & Lab. Invest.*, 4 (1952) 244.
2 K. B. BJORNESJO, *Scand. J. Clin. & Lab. Invest.*, 7 (1955) 153.
3 D. SALKIN, S. H. LAWRENCE, AND H. E. WEIMER, *Diseases Chest*, 31 (1957) 556.
4 S. H. LAWRENCE, H. E. WEIMER, AND D. SALKIN, *J. Lab. Clin. Med.*, 51 (1958) 935.
5 W. P. JENKS, E. R. B. SMITH, AND E. L. DURRUM, *Am. J. Med.*, 21 (1956) 387.
6 G. W. SNEDECOR, *Statistical Methods*, 4th ed., Iowa State College Press, Ames, Iowa, 1946.
7 *Diagnostic Standard and Classification of Tuberculosis*, National Tuberculosis Association, New York, 1955.
8 A. HIRSCH AND C. CATTANEO, *Am. Rev. Tuberc. Pulmonary Diseases*, 75 (1957) 793.
9 F. B. SEIBERT, M. V. SEIBERT, A. J. ATNO, AND H. W. CAMPBELL, *J. Clin. Invest.* 26 (1947) 90.
10 R. W. BALDWIN AND C. N. ILAND, *Am. Rev. Tuberc. Pulmonary Diseases*, 68 (1953) 372.

Received October 15th, 1958

SPECTROPHOTOMETRIC DETERMINATION OF CHROMIUM IN HUMAN PLASMA AND RED CELLS

DWIGHT O. MILLER AND JOHN H. YOE

Pratt Trace Analysis Laboratory, Department of Chemistry, University of Virginia, Charlottesville, Va. (U.S.A.)

The purpose of this investigation was to develop a sensitive, accurate and precise spectrophotometric method for the determination of trace amounts of chromium in human plasma and red cells. The method is based on the absorption properties of the complex formed by the reaction of dichromate with diphenylcarbazide in sulfuric or perchloric acid solution. Chromium (III) is oxidized to dichromate by heating with ammonium persulfate plus a small amount of silver nitrate in 1 *N* sulfuric acid. The high concentration of iron in red cells interferes with the color reaction and is removed by precipitation with cupferron and extraction of the iron-cupferron precipitate into chloroform.

EXPERIMENTAL

Apparatus

(1) *Spectrophotometer.* A Beckman Quartz Spectrophotometer, model DU, equipped with matched 1-cm Corex cells, was used for all absorbance measurements.

(2) *Geiger-Müller counter.* A Tracer Lab binary scaler equipped with an end-window tube.

Reagents

All standard solutions (except potassium dichromate) were stored in polyethylene bottles to prevent possible concentration changes due to adsorption or contamination. Standard dichromate solution is gradually reduced when stored in polyethylene and hence must be stored in glass. All solutions were prepared with chromium-free reagents.

(1) *Chromic chloride.* A stock solution containing 1 mg/ml of chromium was prepared by dissolving 0.5018 g of Matthey's "specpure" chromium metal in hydrochloric acid and diluting to 500 ml with triply-distilled water.

(2) *Dichromate.* A 100 μg/ml solution was prepared by dissolving 0.1514 g of primary standard grade potassium dichromate in triply-distilled water and diluting to 500 ml.

(3) *Chromium-51.* The chromium-51 was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn., as chromic chloride in approximately 1 *N* hydrochloric acid. The specific activity was 32,455 mC/g and the concentration 7.4 $\pm$ 10% mC/ml.

(4) *Diphenylcarbazide reagent solution.* The reagent solution was prepared as follows: 4 g of phthalic anhydride were dissolved in hot 95% ethanol, the solution was then cooled and 0.25 g of diphenylcarbazide was added. The resulting solution was diluted to 100 ml with 95% ethanol and stored under refrigeration to retard decomposition.

References p. 383

Description of the method

The blood samples were drawn, separated and ashed as described by MONACELLI *et al.*[1] After the ashing process, the *plasma ash* is in the form of nitrate salts. These must be converted to the corresponding sulfates because of the adverse effect of the nitrate ions upon the stability of the colored organo-complex. The conversion is accomplished by the addition of 0.5 ml of 7 *N* sulfuric acid to the nitrate salts and heating to fumes of sulfuric acid. The sulfate salts are diluted to approximately 3 ml, giving a solution about 1 *N* in sulfuric acid, and one drop of 1.5 *N* silver nitrate is added. The solution is heated to 90°–95° and about 0.1 g solid ammonium persulfat isa dded; heating is continued for 30 min to complete the oxidation and to decompose the excess oxidizing agent.* Immediately after cooling, the sample is transferred to a 10-ml volumetric flask and 0.5 ml of diphenylcarbazide reagent solution added. The solution is diluted to volume with triply-distilled water and the absorbance measured at 544 mμ. The concentration of chromium is determined from a calibration curve obtained from "synthetic" plasma ash samples (Table I), containing known amounts of chromium, treated in the above manner.

TABLE I

COMPOSITION OF "SYNTHETIC" SAMPLES[a]

Metal ion	*Plasma ash concn p.p.m.*	*Red-cell ash concn p.p.m.*
Na^{+}	2000	1200
K^{+}	200	4000
Ca^{+2}	100	20
Mg^{+2}	20	60
Fe^{+3}	1	500
Cu^{+2}	1	0.9
Zn^{+2}	3	12
Pb^{+2}	0.03	0.6
Mn^{+2}	0.1	0.2
Ni^{+2}	0.02	0.06
Cr^{+3}	(Added separately to individual samples)	

[a] Concentration of the various ions calculated from values given by ALBRITTON[18] or from values determined spectrographically by the Pratt Trace Analysis Laboratory, University of Virginia.

The *red cell ash* is converted to the chlorides and dissolved in 1.2 *N* hydrochloric acid. The solution is cooled to 0°–5° and the iron precipitated by the addition of cupferron. The precipitate is separated by extraction with chloroform. The aqueous layer is evaporated to dryness and the residue converted to its corresponding sulfate salts. From this point on, the procedure is identical to that described for the plasma.

Discussion of the method

EGE AND SILVERMANN[2] and URONE[3] have investigated the behavior of diphenylcarbazide in many solvents and have found that those which are basic cause a rapid decomposition of the reagent but that slightly acid solvents have a stabilizing effect.

* Alternately, the sample may be oxidized by heating with cerium (IV) in 1–5 *N* perchloric acid.

References p. 383

The reagent solution proposed by EGE AND SILVERMANN was used throughout our work. It is colorless at the time of preparation but becomes straw colored upon standing several days. The discoloration has no detrimental effect on either the reagent or the complex and the reagent thus prepared can be used for several weeks.

The color reaction between dichromate and diphenylcarbazide was first observed by GAZENEUVE[4], who postulated that it was due to the formation of an organo-metallic compound. FEIGL[5] noted that the complex was a soluble violet compound of unknown composition, but later[6] assumed that a heteropolyacid might be formed between chromic acid and the reagent. BOSE[7] extensively investigated the nature of the complex and concluded that it was an organo-metallic complex of chromium (II) and diphenylcarbazone.

The complex is formed instantly in the cold if the solution is above 0.01 *N* in sulfuric acid or 0.1 *N* in perchloric acid. Nitric and hydrochloric acids cannot be used since they cause a rapid decomposition of the complex. The acidity of the complex solution is not critical and may be as high as 1 *N* or more. Sulfuric acid is usually recommended[8–11] as the most suitable acid for controlling the acidity of the complex

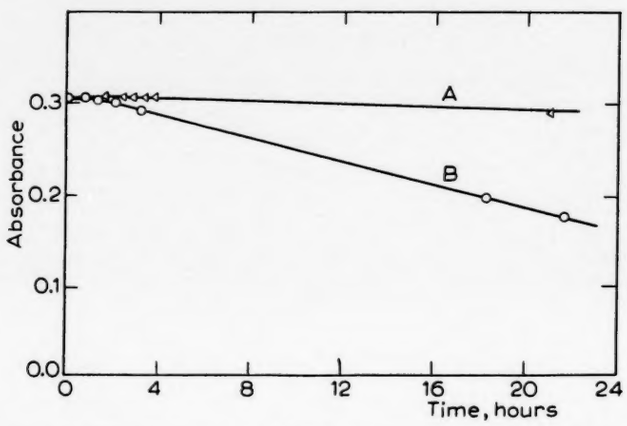


Fig. 1. Stability of chromium complex. A. In 0.12 *N* perchloric acid. B. In 0.09 *N* sulfuric acid.

solution; however, our investigation revealed that the use of perchloric acid gives a more stable complex. Sulfuric acid solutions of the complex begin to fade appreciably after about 1 1/2 h but perchloric acid solutions exhibit the same absorbance for more than 4 h after formation of the complex; even after 28 h the absorbance had decreased only about 7% (see Fig. 1).

The color system obeys Beer's law over a concentration range of 0.01 to 1.0 p.p.m. The optimum range of absorbance measurements as defined by SANDELL[12] (A = 0.2 to 0.7) corresponds to a chromium concentration of 0.3 to 1.1 p.p.m.

Diphenylcarbazide reacts with many ions[13] to form colored complexes: however, in red cells the only interference is from iron; in plasma the iron concentration is so low that it causes no interference. None of the other metallic constituents of blood are present in concentrations high enough to give any detectable interference. Iron was separated from the red cell samples by precipitation with cupferron and extraction of the precipitate with chloroform from 1.2 *N* hydrochloric acid, which is reported to remove even spectrographic traces of iron[14]. It is necessary to cool the solution to 0°–5° because of the tendency of cupferron to decompose with heat into nitrosobenzol[15]. The distribution of the chromium between the two phases was determined

References p. 383

by a tracer technique using radioactive chromium-51. The isotope was added to "synthetic" red cell ash solution (Table I), the cupferron precipitation was made and the extraction then carried out. After each extraction, an aliquot was taken from each phase and the γ radiation (due to the electron capture decay of the chromium-51) measured with an end-window Geiger tube which was attached to a binary scaler. The ratio of the count rates of the organic and acid phases gives the chromium distribution without further refinement. The chromium content of the organic phase was so low that it could not be determined with any statistical certitude. From the results in Table II it is seen that 99 to 100% of the chromium was retained in this acid phase.

TABLE II

PHASE DISTRIBUTION OF CHROMIUM

Sample No.	*Counts/min acid phase*[a]	*Counts/min organic phase*[a]
1	1627	39
2	845	36
		33 second ext.
3	847	37

[a] Uncorrected for background which was 36 counts/min. If the organic phase counts are corrected for background, obviously the chromium content is nil. The total number of counts in each case was large enough so that the expected accuracy would be of the order of $\pm$ 5%.

Excess oxidizing agent will oxidize the diphenylcarbazide reagent and cause a rapid decomposition of the organo-metallic complex. The persulfate oxidation has the advantage, that the excess oxidizing agent is decomposed by heating. Investiga-

RESULTS

TABLE III

DATA ON THE ACCURACY AND PRECISION "SYNTHETIC" PLASMA ASH[a]

Sample No.	*Absorbance*	*d*	d^2
1	0.600	0.007	0.000049
2	0.610	0.003	0.000009
3	0.610	0.003	0.000009
4	0.622	0.015	0.000225
5	0.616	0.009	0.000081
6	0.595	0.012	0.000144
7	0.616	0.009	0.000081
8	0.586	0.021	0.000441
9	0.603	0.004	0.000016
10	0.613	0.006	0.000036
11	0.610	0.003	0.000009
	6.681		$\Sigma d^2 = 0.001100$
	0.607 (Average)		

$$\text{Standard Deviation} = \sqrt{\frac{\Sigma d^2}{n-1}} = \sqrt{\frac{0.0011}{10}} = 0.01$$

$$\frac{0.01 \times 100}{0.607} = 1.6\% \text{ (for one measurement)}$$

[a] These samples contained 1μg of chromium per ml and the absorbance average of 0.607 unit corresponded to 0.99 μg/ml, equivalent to a 99% recovery.

References p. 383

tion of this oxidation process has shown that more consistent results are obtained by keeping the temperature below the boiling point of the solution.

TABLE IV

DATA ON ACCURACY AND PRECISION "SYNTHETIC" RED CELL ASH[a]

Sample No.	*Volume of sample*	*Cr added p.p.m.*	*Cr found p.p.m.*	*Deviation p.p.m.*
1[b]	10 ml	1.00	0.97	0.03
2[b]	10	1.00	0.97	0.03
3[b]	10	1.00	0.92	0.08
4[b]	10	1.00	1.00	0.00
5[b]	10	1.00	0.96	0.04
6[b]	10	1.00	0.96	0.04
7[b]	10	1.00	0.96	0.04
8	10	1.00	0.92	0.08
9	10	1.00	0.96	0.04
10	10	1.00	0.99	0.01
11	10	1.00	0.97	0.03
12	10	1.00	0.97	0.03

Average recovery, 97% Average deviation, ± 0.04 p.p.m.
[a] Results are recorded as w/v concentrations.
[b] These samples were oxidized with cerium (IV) in pherchloric acid.

TABLE V

ANALYSIS OF HUMAN PLASMA

Sample No.[a]	*Cr found p.p.m.*	*Cr found spectrographically*[b] *p.p.m.*
A 2, P–2	0.052	——
151, P–2	0.027	0.028
156, P–2	0.030	0.032
158, P–2	0.029	0.032
159, P–2	0.034	——
162, P–1	0.026	0.026
183, P–1	0.017	0.017
184, P–1	0.026	0.025

[a] Pratt Trace Analysis Laboratory sample numbers.
[b] Analyses by LEONEL M. PAIXAO, Research Associate, Pratt Trace Analysis Laboratory.

TABLE VI

ANALYSIS OF HUMAN RED CELLS

Sample No.[a]	*Cr found p.p.m.*	*Cr found spectrographically*[b]
181, C–1	0.015	0.016
182, C–1	0.038	0.016
183, C–1	0.014	0.011
184, C–1	0.024	0.016
194, C–1	0.030	——
197, C–1	0.021	——

[a] Pratt Trace Analysis Laboratory sample numbers.
[b] Analyses by LEONEL M. PAIXAO, Research Associate, Pratt Trace Analysis Laboratory.

References p. 383

A large number of "synthetic" plasma and red cell ash samples containing known amounts of chromium were analyzed in order to determine the accuracy and precision of the method. The results are summarized in Tables III and IV.

The concentration of chromium found in normal human plasma ranged from 0.017 to 0.052 p.p.m. (See Table V) and is in good agreement with the values reported by KOCH *et al.*[16] and SHIMP *et al.*[17] whose values ranged from 0.007 to 0.052 p.p.m. The chromium concentration in red cells ranged from 0.014 to 0.038 p.p.m. (See Table VI).

The accuracy of the method is shown by the results obtained from analysis of synthetic samples and by the agreement of our results with those found spectrographically on the same samples.

ACKNOWLEDGEMENT

The blood samples were collected by Mrs. JOYCE MAHON, technician in the Hematology Laboratory of the University of Virginia School of Medicine. We wish to express our thanks for this assistance.

SUMMARY

A spectrophotometric method is presented for the determination of traces of chromium in human plasma and red cells. It is based upon the red-violet complex formed by the reaction of dichromate with diphenylcarbazide and includes an oxidation process for the conversion of chromium (III) to dichromate and a separation of iron (III) from chromium (III). Data on the accuracy and precision of the method are given. Values found for chromium in the normal blood fractions are compared with those reported in the literature. The method described for red cells can be applied to whole human blood without modification.

REFERENCES

1 R. MONACELLI, H. TANAKA AND J. H. YOE, *Clin. Chim. Acta*, 1 (1956) 577. Correction: The equation on page 579 should read $r = 1/c \sqrt{\Sigma d^2/n}$.
2 J. F. EGE, JR. AND L. SILVERMAN, *Anal. Chem.*, 19 (1947) 693.
3 P. F. URONE, *Anal. Chem.*, 27 (1955) 1354.
4 P. CAZENEUVE, *Compt. rend.*, 131 (1900) 346.
5 F. FEIGL, *Spot Tests*, 3rd ed., Elsevier, Amsterdam, 1946, p. 128.
6 F. FEIGL, *Chemistry of Specific, Selective and Sensitive Reactions*, Academic Press, Inc., New York, 1949, p. 242.
7 M. BOSE, *Anal. Chim. Acta*, 10 (1954) 201.
8 H. J. COHNMAN AND R. BISEN, *Anal. Chem.*, 24 (1952) 1341.
9 J. F. WELCHER, *Organic Analytical Reagents*, Vol. III, Van Nostrand, New York, 1947, p. 434.
10 G. ROWLAND, *Ind. Eng. Chem., Anal Ed.*, 11 (1939) 442.
11 P. F. URONE AND H. K. ANDERS, *Anal. Chem.*, 22 (1950) 1317.
12 E. B. SANDELL, *Colorimetric Determination of Traces of Metals*, 2nd ed., Interscience Publ., Inc., New York, 1950 p. 64.
13 G. GUTZEIT, *Helv. Chim. Acta*, 12 (1929) 715.
14 N. H. FURMAN, W. B. MASON AND J. S. PEKOLA, *Anal. Chem.*, 21 (1949) 1325.
15 G. FREDERICK SMITH, *Cupferron and Neo-cupferron*, The G. Frederick Smith Chemical Co., Columbus, Ohio, 1939.
16 H. J. KOCH, JR., E. R. SMITH, N. F. SHIMP AND J. CONNER, *Cancer*, 9 (1956) 449.
17 N. F. SHIMP, J. CONNER, A. I. PRINCE, F. E. BEAR, H. J. KOCH, JR. AND E. R. SMITH, *Methods and Results of Spectrographic Analysis of Human Blood and Tissues*, Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy, March 4, 1955.
18 E. C. ALBRITTON, *Standard Values in Blood*, W. B. Saunders Co., Philadelphia, 1952, pp. 117–119.

Received August 1st, 1958

A SIMPLE COLORIMETRIC METHOD FOR ESTIMATING SERUM PSEUDOCHOLINESTERASE

R. L. SMITH AND H. LOEWENTHAL

Department of Pathology, Chase Farm Hospital, Enfield, Middlesex (Great Britain)

and

H. LEHMANN AND ELIZABETH RYAN

Department of Pathology, St. Bartholomew's Hospital, London (Great Britain)

Blood contains 2 enzymes which are capable of hydrolysing acetyl choline. One, the "true" cholinesterase is contained in the red cells, while the other, the "pseudo" cholinesterase is found in the serum. Among the points of difference between these 2 enzymes is the ability of pseudocholinesterase to hydrolyse certain non-choline esters, such as ethyl acetate and phenyl benzoate.

The estimation of serum pseudocholinesterase has two main uses in clinical pathology. It is of value in detecting poisoning by organic phosphorus insecticides such as Parathion, since a lowering of the serum pseudocholinesterase level precedes the onset of symptoms, (GALLEY *et al.*[1], GAGE[2]). It is also accepted that a low serum pseudocholinesterase level can be an indication of lowered liver function, (MCARDLE[3], WESCOE *et al.*[4], VORHAUS *et al.*[5, 6], KAUFMAN[7], HUNT AND LEHMANN[8], and many others).

Numerous methods for the estimation of serum pseudocholinesterase have been devised. References of 24 of them are given by GOMORI[9].

RIDER *et al.*[10] used the method of GOMORI[11] in which the esterase activity was measured by incubating the enzyme for 1 hour at 37° with a pH 6.3 phosphate buffer containing phenyl benzoate. The amount of phenol liberated was measured by a colour reaction with a stable diazotate, Fast Red B.

This method did not give reliable results in our hands, due mainly to the variability of the colour reaction, so we have elaborated another pseudocholinesterase estimation which uses the same substrate, phenyl benzoate. The technique has been compared with the measurement of enzyme activity by the micro manometric technique using the Warburg apparatus (MCARDLE[3]). This depends on the evolution of CO_2 from a bicarbonate buffer by the products of ester hydrolysis. The activity of the enzyme is expressed as μl of CO_2 formed per minute by 1 ml of serum or plasma at 37°, here briefly called 'Warburg Units'.

METHOD

Reagents

(1) *Buffer*, pH 8.6, 0.1 *M*. 260 ml of 0.1 *N* HCl are added to 1740 ml of 0.1 *M* sodium barbitone (20.6 g/l.). A pH of 8.6 is achieved if necessary by adding 0.1 *M* sodium barbitone or 0.1 *N* HCl. If stored in a refrigerator, this buffer is stable for many months.

(2) *Tween 80*, 100 mg/100 ml in water. This will keep for a least a month at room temperature.

References p. 390

(3) *Phenyl benzonate*, 2% in methanol. This will keep for a least a month in the refrigerator.

(4) *Buffered substrate.* 100 ml buffer are brought to 37°, 1 ml Tween 80 solution is added, followed just before use by 0.15 ml of phenyl benzoate solution.

(5) *Sodium chloride* 20% in water
(6) FOLIN AND CIOCALTEU'S *reagent*
(7) *Standard phenol-plus-reagent*
(8) *Sodium carbonate* 15% (anhydrous)

} As in KING[12]

Procedure

The amounts given are those required when a "Spekker" absorptiometer with 2-cm cups is used at the end. If an instrument is used where a final volume of 9 ml is sufficient, the amounts given may be halved throughout.

Add 0.5 ml serum to 9 ml 20% sodium chloride solution; dilute to 200 ml with water and mix. Alternatively dilute 0.1 ml of serum to 40 ml with physiological saline.

Take 2 tubes: "Test" and "Control". Into "Test" place 1 ml diluted serum. Warm both tubes to 37°, add 10 ml buffered substrate brought to the same temperature to both tubes and mix tube "Test". Exactly 60 minutes after adding the buffered substrate, add 1 ml of undiluted phenol reagent to each tube and mix. Add 1 ml diluted serum to "Control" and mix. In a third tube "Standard" mix: 6 ml standard phenol-plus-reagent, 0.5 ml undiluted phenol reagent and 5.5 ml water. To each tube add 6 ml 15% sodium carbonate, mix, and incubate at 37° for 10 min. If any turbidity forms at this stage, it should be removed by centrifuging. Take the readings of all 3 tubes in a photo-electric absorptiometer, using a red filter.

Then

$$\frac{\text{Test-Control}}{\text{Standard}} \times 60 = \mu\text{g phenol liberated}$$

$$\frac{\text{Test-Control}}{\text{Standard}} \times 150 \equiv \text{serum pseudocholinesterase (in Warburg units)}$$

If the serum pseudocholinesterase is above 150 Warburg units, the estimation should be repeated using a greater dilution of serum and the calculation altered accordingly.

FACTORS GOVERNING THE CHOICE OF REAGENTS AND CONDITIONS

1. *Colour reagent*

The phenol reagent of FOLIN AND CIOCALTEU gave more reliable results than Fast Red B, which was used by RIDER *et al.*[10]. The FOLIN AND CIOCALTEU reagent has the additional advantage of being widely used for phosphatase estimations and is therefore readily available in most laboratories.

2. *Substrate*

Phenyl benzoate is not an ideal substrate for enzyme estimation because of its low solubility in water (about 40 mg/l). Attempts to increase the solubility of phenyl benzoate by using dilute alcohol or acetone led to inhibition of the enzyme activity or interference with the colour reaction.

The use of other substrates was considered. Naphthyl acetates, which have been used for esterase detection in histochemistry, could not be used because naphthol, the

References p. 390

product of the enzyme action, was not suitable for reaction with CIOCALTEU's reagent.

MOUNTER AND WHITTAKER[13] found that human plasma hydrolysed phenyl acetate at a high rate, but that this hydrolysis was inhibited only 12% by 10 μM eserine. As eserine inhibits pseudocholinesterase at this concentration, most of the hydrolysis of phenyl acetate was therefore due to an unspecific esterase activity.

Phenyl *n*-butyrate is more soluble in water than phenyl benzoate and is hydrolysed by serum, the optimum pH being 8.4. This hydrolysis was largely suppressed by prostigmin bromide and a comparison of the serum esterase activity estimated with this substrate and the serum pseudocholinesterase estimated by the Warburg method showed a reasonable degree of correlation. However, non-enzymic hydrolysis of the phenyl *n*-butyrate gave rather high blank values.

In view of these facts, phenyl benzoate was chosen as the most suitable substrate in spite of its low solubility.

3. *pH*

pH 8.6 was chosen because maximum activity of pseudocholinesterase was found at this pH. The non-specific esterase has very little activity anyhow, but it is about the same at pH 6.3 and pH 8.6, hence, compared with pseudocholinesterase, its effect will interfere least at pH 8.6. RIDER *et al*[10] had used a pH of 6.3 because GOMORI[11] found that this was the optimum pH for the action of pancreatic extract on phenyl benzoate. We estimated the esterase activity of 47 sera, using phenyl benzoate at pH 6.3, but the correlation with the Warburg manometric technique was only moderate, whereas it is satisfactory at pH 8.6.

4. *Specificity of substrate*

GOMORI[14] commented on the possibility of using non-choline substrates for estimating serum pseudocholinesterase and suggested the use of a control tube containing eserine as a safeguard of specificity. The eserine preparations readily available gave rather high blank values, so prostigmin bromide was used instead. At pH 6.3 a final concentration of 33 mg/l gave maximum inhibition. At this pH when the proportion of non specific enzyme activity is not as insignificant as it is at pH 8.6, consideration of the prostigmin sensitive esterase improved the correlation with the pseudocholinesterase values obtained by the Warburg technique (Fig. 1). The prostigmin inhibition was variable, and in a few instances esterase activity in the presence of prostigmin was considerable. There was no clinical feature common to these cases. This work was carried out some years ago, before KALOW AND STARON[15] published their findings on the variable effect of dibucaine on the hydrolysis of benzoylcholine by pseudocholinesterase. It now seems likely that we were dealing with the genetical differences in pseudocholinesterase type discovered by the Canadian workers.

5. *Tween 80*

The addition of 1 mg Tween 80 per 100 ml buffer assisted the solution of phenyl benzoate considerably. If the Tween 80 was omitted, the phenyl benzoate often formed a scum on the surface of the buffer which was very difficult to dissolve.

6. *Amount of serum and time of incubation*

If the amount of serum was increased and the incubation period correspondingly

References p. 390

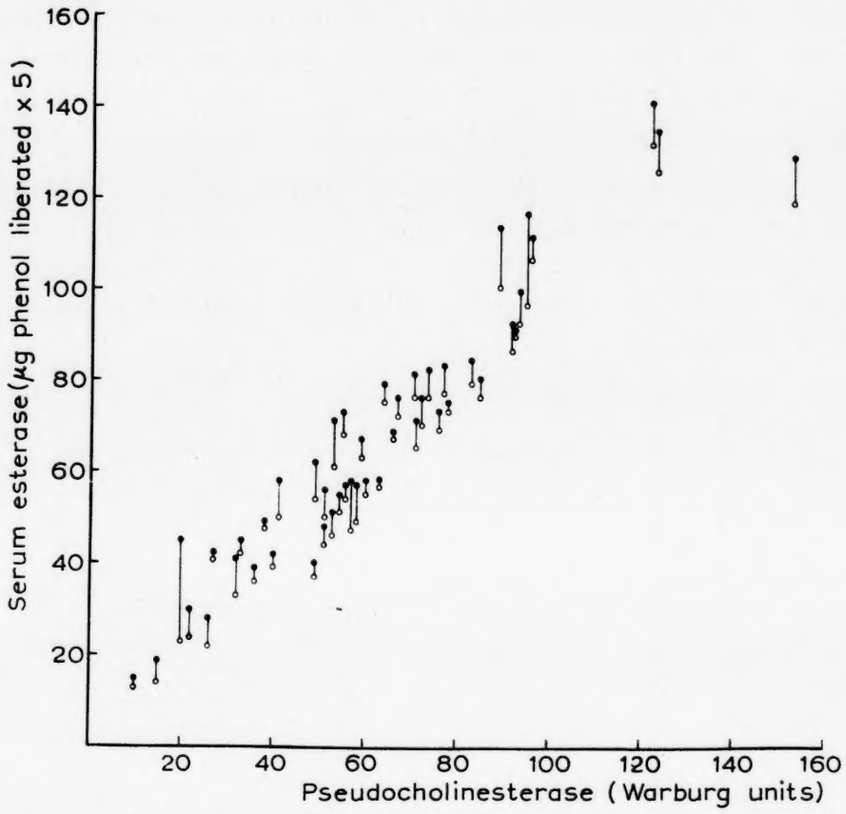


Fig. 1. Prostigmin sensitive esterase. ● Total esterase; ○ Prostigmin sensitive esterase. The lengths of the vertical lines show the amounts of prostigmin insensitive esterase. In the serum esterase estimations, 10 μl serum hydrolysed phenyl benzoate at pH 6.3 for 1 h at 37°.

lessened, the blank values, due to the protein in the serum, became rather high. Much longer incubation periods also tended to increase the blank values because of non-enzymic hydrolysis of the substrate at pH 8.6.

RELIABILITY OF THE METHOD

A number of investigations were carried out to test the reliability of the method.

1. *Colour reaction*

The presence of diluted serum, phenyl benzoate, buffer, Tween 80 and prostigmin bromide did not affect the colour reaction and a quantitative recovery of phenol was obtained in the presence of all of these reagents.

2. *Tween 80*

The addition of up to 3 mg Tween 80 per 100 ml of buffer did not affect the results of the estimations.

3. *Reproducibility*

Duplicate estimations carried out on a single dilution of serum showed a mean difference of 1.4 units (190 sera). Duplicate estimations carried out on separate dilutions of serum on different days showed a mean difference of 2.6 units (48 sera). These results were obtained with 2-cm cups in the "Spekker" absorptiometer. Under these conditions, a difference of 0.01 in the optical densities of the test and control corres-

References p. 390

ponds to about 2 Warburg units. Using the Unicam Sp. 300, the Hilger Biochem Absorptiometer or the EEL portable colorimeter, with round cuvettes, a difference in optical density of 0.01 corresponds to about 3 Warburg units.

4. *Enzyme activity of different amount of serum*

When the amounts of phenol liberated by different dilutions of the same serum were estimated, it was found that they were proportional to the amount of serum present up to a liberation of 60 μg phenol (equivalent to 150 Warburg units of pseudocholinesterase) (see Fig. 2). This corresponds to the hydrolysis of about 40% of the phenyl benzoate present.

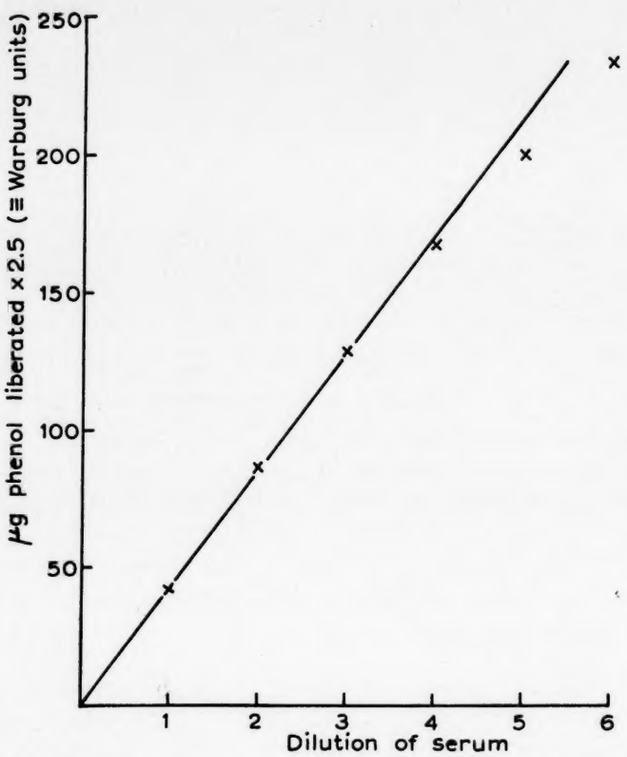


Fig. 2. Effect of dilution on the estimation of esterase. Liberation of phenol by different amounts of serum. 1 ml of diluted serum hydrolysed phenyl benzoate at pH 8.6 for 1 hour at 37°.

5. *Comparison with the Warburg method*

The present method was compared with the Warburg pseudocholinesterase values in 200 sera. The results are shown in Fig. 3. The average difference between the 2 results was 6 units, and the standard deviation of the differences 7.6 units.

6. *The effect of prostigmin*

In 38 sera, particularly those in which the esterase value was higher than the Warburg pseudocholinesterase result, the effect of prostigmin was noted. A final concentration of 19 mg prostigmin bromide per litre was found to give complete inhibition of esterase activity (pH 8.6) in every case.

7. *The effect of haemolysis, heparin and storage of serum*

Haemolysis of the serum did not affect the result, and washed haemolysed red cells had no appreciable esterase activity. Large amounts of heparin (300 units/ml of

References p. 390

blood) lowered the result slightly. It seems likely that moderate amounts of heparin will not affect the results, but serum has been used throughout this work.

The serum pseudocholinesterase is stable for several days at room temperature and for months at —20°.

DISCUSSION

In spite of many reports of the usefulness of serum pseudocholinesterase estimations, this test has not been very widely used. One reason for this is the lack of a generally accepted simple method. The colorimetric method of DE LA HUERGA *et al.*[16] was applied to 18 sera in parallel with the Warburg method and there was good agreement. Taking 2 units of the colorimetric method to be equivalent to 1 Warburg unit, the maximum difference between the 2 methods was 10 Warburg units and the average difference 4.5 units. It was felt, however, that this colorimetric method was too tedious for routine use in a busy general laboratory.

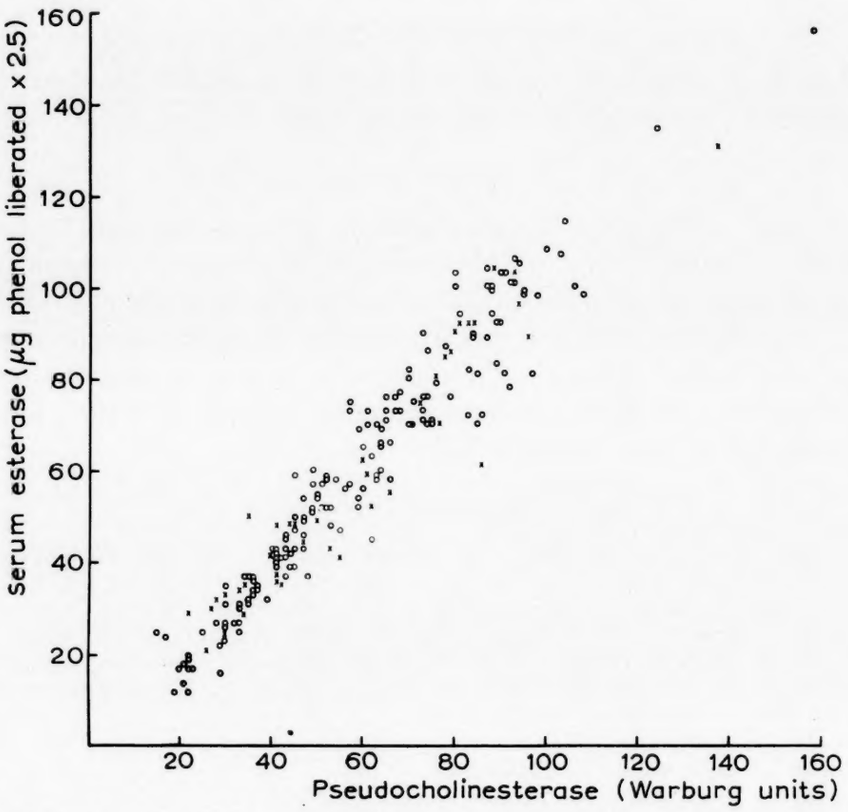


Fig. 3. Comparison of colorimetric estimation of esterase with manometric determination of pseudocholinesterase. × Esterase estimated at St. Bartholomew's Hospital; ○ Esterase estimated at Chase Farm Hospital. In 38 sera tested there was no difference between the total and prostigmin sensitive esterase values. In the serum esterase estimations, 2.5 μl serum hydrolysed phenyl benzoate at pH 8.6 for 1 h at 37°.

The Warburg technique is generally accepted as a reliable method, but it requires expensive apparatus and skilled technique. Nevertheless it is by this method that the normal range of pseudocholinesterase values and the variations found in disease have been most widely studied. For this reason the method described in this article has been compared with the Warburg method in 200 sera. Bearing in mind the

References p. 390

complete difference between the two methods, the degree of correlation shown in Fig. 3 seems to be satisfactory.

As the method described uses a non-choline substrate, it is important to be sure that it is in fact pseudocholinesterase which is being measured. The complete inhibition of esterase activity at pH 8.6 by prostigmin in all of the 38 sera tested is therefore important.

The present method has been used without difficulty by comparatively junior laboratory technicians. It requires no more skill than the estimation of serum phosphatase by the KING-ARMSTRONG technique of which it is in fact a variation. As the amount of serum used in the test in only 5 μl, the method seems particularly suited to work on infants. The actual amount of serum required will in fact depend on the smallest volume of serum which can be measured accurately.

Sera giving results above 150 Warburg units must be retested with more dilute serum but this is not a great disadvantage, as values over 150 units are not at all common.

ACKNOWLEDGEMENT

We should like to thank Miss VALERIE PATSTON, Research Assistant, St. Bartholomew's Hospital for her help with some of the work.

SUMMARY

A simple colorimetric method for estimating serum pseudocholinesterase is presented. It depends on the hydrolysis of phenyl benzoate at pH 8.6 by the serum with the subsequent determination of the liberated phenol by the use of FOLIN AND CIOCALTEU's reagent. The evidence that the results are a measure of serum pseudocholinesterase activity is (1) complete inhibition of enzymic hydrolysis by prostigmin and (2) a comparison between the serum esterase activity and the pseudocholinesterase as determined by the Warburg technique.

REFERENCES

1 R. A. E. GALLEY, D. R. DAVIES AND P. L. BIDSTRUP, *Proc. Roy. Soc. Med.*, 45 (1952) 567.
2 J. C. GAGE, *Brit. Med. J.*, 1 (1955) 1370.
3 B. MCARDLE, *Quart. J. Med.*, 9 (1940) 107.
4 W. C. WESCOE, C. H. HUNT, W. F. RIKER AND I. C. LITT, *Am. J. Physiol.*, 149 (1947) 549.
5 L. J. VORHAUS, H. H. SCUDAMORE AND R. M. KARK, *Gastroenterology*, 15 (1950) 304.
6 L. J. VORHAUS, H. H. SCUDAMORE AND R. M. KARK, *Am. J. Med. Sci.*, 221 (1951) 140.
7 K. KAUFMAN, *Ann. Intern. Med.*, 41 (1954) 533.
8 A. H. HUNT AND H. LEHMANN, *Clin. Chem.*, 2 (1956) 251.
9 G. GOMORI, *Am. J. Clin. Pathol.*, 24 (1954) 99.
10 J. A. RIDER, H. C. MOELLER AND K. P. DU BOIS, *Proc. Soc. Exptl. Biol. Med.*, 76 (1951) 427.
11 G. GOMORI, *J. Lab. Clin. Med.*, 34 (1949) 275.
12 E. J. KING, *Micro-analysis in Medical Biochemistry*, 3rd ed., Churchill, London, 1956.
13 L. A. MOUNTER AND V. P. WHITTAKER, *Biochem. J.*, 54 (1953) 551.
14 G. GOMORI *J. Lab. and Clin. Med.*, 42 (1953) 445.
15 W. KALOW AND N. STARON, *Can. J. Biochem. and Physiol.*, 35 (1957) 1305.
16 J. DE LA HUERGA, C. YESINICK AND H. POPPER, *Am. J. Clin. Pathol.*, 22 (1952) 1126.

Received October 22th, 1958

THE EFFECT OF FACTORS CONTAINED IN THE MEMBRANES OF RED BLOOD CELLS ON THE SHAPE OF THE DISSOCIATION CURVE OF HEMOGLOBIN

J. HOŘEJŠÍ AND A. KOMÁRKOVÁ

Institute of Hematology and Blood Transfusion and Laboratory of Protein Metabolism of the Medical Faculty, Charles University, Prague (Czechoslovakia)

In our previous study[1] we showed that the shape of the dissociation curve of hemoglobin could be considerably influenced by free SH-groups, *e.g.* by glutathion. By raising the content of SH-groups, HILL's constant is lowered and the curve shifts to the right. On blocking the SH-groups, *e.g.* by mercury, the opposite result is obtained.

We then considered the behaviour of pure crystalline hemoglobin under similar conditions. For the preparation of human hemoglobin the method described by DRABKIN[2] was used.

From Table I and Fig. 1 it follows that the affinity of the solution of pure hemoglobin to oxygen was not influenced by glutathion as in the case of whole blood.

In our recent experiments we closely studied the hemolysates of red blood cells and, in addition the effect of the membranes of red blood cells on the dissociation curve of pure hemoglobin. The results are shown in Table I and Fig. 1. They reveal,

TABLE I

	Hill's constant		*Hill's constant*
Native blood	0,000279	Solution of crystalline hemoglobin + membranes of red blood cells	0.000132
Hemolysate	0.000332		
Hemolysate + glutathion	0.000205	Solution of crystalline hemoglobin + membranes of red blood cells + glutathion	0.000104
Solution of crystalline hemoglobin	0.000221		
Solution of crystalline hemoglobin + glutathion	0.000217		

that the SH-groups influenced the shape of the dissociation curve of blood hemolysate in a manner similar to that of whole native blood. We added to the solution of the crystalline hemoglobin—in which the effect of glutathion on the oxygen-binding capacity of hemoglobin could not be detected—the membranes of red blood cells, isolated from hemolysate by centrifugation at 20,000 r.p.m. After the addition of these membranes, HILL's constant of hemoglobin changed at once. We also observed, that in this system, the effect of glutathion on the shape of the dissociation curve is evident in native blood as well as in full hemolysate.

From these experiments it can be seen that the position of the dissociation curve of hemoglobin is significantly influenced—in addition to conditions generally known—by factors pertaining to the membranes and stroma of red blood cells. At first we studied carbonic anhydrase, which exists in the erythrocytes in high concentration and high activity. Carbonic anhydrase can be blocked with cyanates and certain sulphonamides. For this purpose we used Diamox. The solution was prepared with

References p. 392

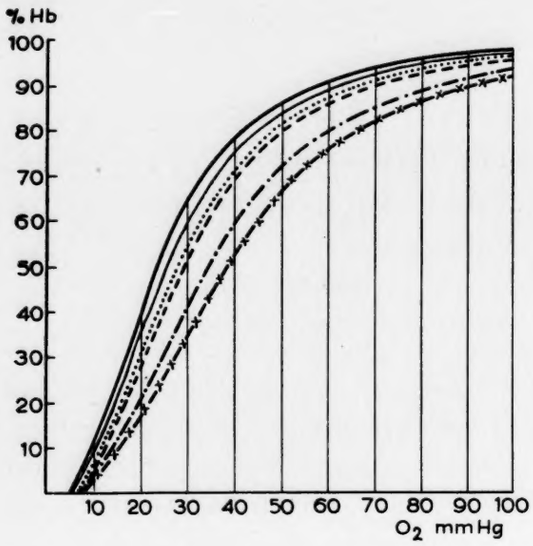


Fig. 1. ——————— Hemolysate; — — — — — — Hemolysate + glutathion; ——————— Native blood; —.—.—.—.— Sol. of cryst. hemoglobin + membranes of erythrocytes; —×—×—×— Sol. of cryst. hemoglobin + membranes of erythrocytes + glutathion; . . . . . Sol. of crystalline hemoglobin, sol. of crystalline hemoglobin + glutathion (both curves are identical).

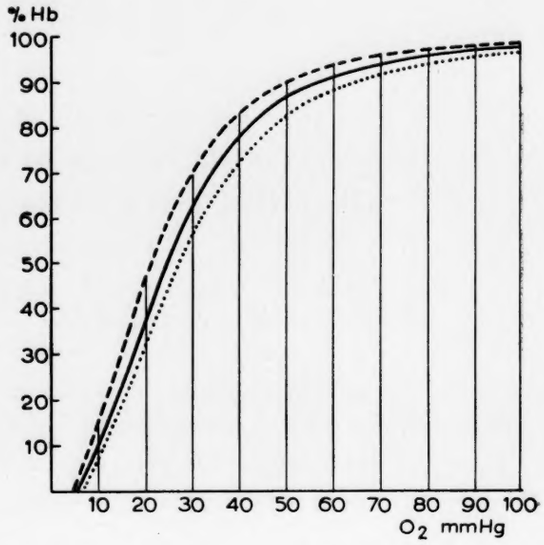


Fig. 2. ——————— Native blood; . . . . . Native blood + glutathion; – – – – – – – Native blood + Diamox.

one-half tablet of Diamox, *i.e.* 125 ml, in 5 ml of 0.9% saline solution. The insoluble materials of the tablet were centrifuged and 1 ml of the supernatant was added to 10 ml of blood. The result, Table 2 and Fig. 2, indicated that on blocking the carbonic anhydrase with Diamox, the position of the dissociation curve changes; *i.e.*, the left-side shift appears. The effect is the same as the blocking of SH-groups by heavy metal (Hg).

TABLE II

	Hill's constant
Native blood	0.000336
Native blood + glutathion	0.000254
Native blood + Diamox	0.000463

From this experiment it seems probable that the carbonic anhydrase is not only important for the transportation of carbon dioxide by the red blood cell (Bohr's effect), but that this enzyme also plays an important role in the transportation of oxygen by hemoglobin. The mechanism of this effect will be studied further.

SUMMARY

The dissociation curve of crystalline hemoglobin is not influenced by glutathion, in contrast to that of native blood. The effect of glutathion appears after the addition of the stroma of red blood cells. The dissociation curve is probably influenced by factors contained in this stroma. Carbonic anhydrase, the blocking of which significantly influences the shape of the dissociation curve, may be one of these factors.

REFERENCES

[1] J. HOŘEJŠÍ AND A. KOMÁRKOVÁ, *Clin. Chim. Acta*, 3 (1958) 131.
[2] D. L. DRABKIN, *Arch. Biochem.*, 21 (1949) 224.

Received May 7th, 1958

ARTERIOVENOUS DIFFERENCE OF CALCIUM AND CITRIC ACID IN HYPERPARATHYROIDISM

O. BLEHA, A. KOMÁRKOVÁ, J. VOSTÁL, V. PACOVSKÝ, E. VITKOVÁ,

IIIrd Internal Clinic and Central Biochemical Laboratories, Medical Faculty Hospital, Charles University, Prague (Czechoslovakia)

Striking oscillation of the serum calcium between normal and elevated levels in hyperparathyroidism is a well-known fact indicating profound dynamic differences in calcium metabolism. This may be due to the irregular elimination of the parathyroid hormone from the tumor. During metabolic examinations performed in our patients and during the studies of the renal elimination of calcium and citric acid, we studied the arteriovenous difference of calcium and citric acid levels in 9 normal individuals, in 5 patients with different disorders of calcium metabolism or with metabolic bone disease and in 6 patients with hyperparathyroidism. In all these patients bone, biochemical, operative and histological examination confirmed the diagnosis. In 4 cases, adenoma of the parathyroid gland was found, in 1 case the nodular hyperplasia of parathyroids was histologically established.

METHOD

Blood was taken before breakfast simultaneously from the arteria femoralis and vena cubitalis. The calcium level from superficial veins of the leg proved to be the same as in the vena cubitalis. For the determination of calcium we used the method of CLARK AND COLLIP[1], citric acid was determined according to GEY[2].

RESULTS

The results of the examination are shown in Table I. In all 9 controls, calcium was within the range of normal levels and no significant arteriovenous difference could be demonstrated. The maximal difference of 2% lies within the range of laboratory error. In a further group of 5 patients, only one significant difference (4%) was found in a patient with tetania strumipriva and low calcium level. Striking arteriovenous differences of 12–16% were found in all cases of hyperparathyroidism, while in the patient with hyperplastic parathyroid glands, the arteriovenous difference was lower (5%). These differences could not be explained by the errors of the method, because a vast clinical material (more than 100,000 samples) was examined by this method and the difference of the simultaneous determinations never exceeded the range of $\pm$ 0.1 mg %.

Citric acid level is always higher in venous blood and the arteriovenous difference with the maximum of 35% shows no significant deviation between the observed groups.

References p. 394

TABLE I

	Diagnosis	Calcium mg %		Citric acid mg %	
		vein	*artery*	*vein*	*artery*
K.V.	Adenoma of the parathyr. gland	15.8	17.2	6.0	5.8
E.F.		12.1	13.9	–	–
V.P.		12.4	14.0	5.4	3.5
G.H.		10.2	12.0	9.4	8.4
H.E.		13.7	11.0	4.9	4.2
M.F.	Nodular hyperplasia of the parathyr. gland	9.6	10.1	2.8	2.3
L.S	Normal	9.5	9.6	2.8	1.8
J.N.		10.9	11.0	1.8	1.2
A.B.		9.9	9.8	1.7	1.4
E.K.		9.2	9.2	1.9	1.4
F.E.		9.2	9.2	–	–
L.H.		9.7	9.6	2.0	2.0
V.H.		9.0	9.0	2.1	1.4
O.H.		9.3	9.4	2.4	2.–
L.V.		10.2	10.0	2.7	2.–
A.C.	Osteoporosis	10.–	9.9	2.7	1.9
F.K.	Osteomalacia	10.–	9.8	2.5	1.8
L.T.	Hyperventilatory tetania	9.7	9.9	1.2	1.2
M.S.	AT 10 overdosing	12.–	12.1	2.8	2.2
E.P.	Tetania strumipriva	6.8	7.1	2.5	1.9

SUMMARY

1. In 6 patients with hyperparathyroidism a striking arteriovenous difference of serum calcium has been found.

2. In 9 normal individuals and in 5 patients with other disorders of calcium metabolism or bone disease except one case of tetania strumipriva no arteriovenous difference of serum calcium could be detected.

3. This observation—for which no satisfactory explication has yet been found—is of interest for pathophysiological studies and for the clinical praxis because the arterial calcium level seems to be—in some cases—a better indicator of metabolic disorders in hyperparathyroidism than the level in venous blood.

4. No significant arteriovenous difference could be traced in the level of citric acid in hyperparathyroidism as compared with the controls.

REFERENCES

[1] E. P. CLARK AND J. B. COLLIP, *J. Biol. Chem.*, 63 (1925) 461.
[2] K. F. GEY, *Intern. Z. Vitaminforsch.*, 25 (1953) 21.

Received September 25th, 1958

AN IMPROVED GLUCOSE-OXIDASE METHOD FOR DETERMINING BLOOD, C.S.F. AND URINE GLUCOSE LEVELS

VINCENT MARKS

Dept. of Chemical Pathology, Institute of Neurology, The National Hospital, London (Great Britain)

The introduction by FROESCH AND REYNOLDS[1] of an enzyme method for the determination of glucose levels in biological fluids gave clinical biochemists the first specific method for determining this substance. However, despite its ease of performance the time consumption was so great as to make it impracticable for routine hospital laboratory work. Later in the year KESTON[2] and TELLER[3] suggested the use of a coupled enzyme system in which the hydrogen peroxide formed in the enzymic oxidation of glucose was destroyed by peroxidase in the presence of a suitable chromogenic oxygen acceptor. The chromogen being in direct proportion to the amount of glucose originally present, could then be determined colorimetrically.

Following this suggestion, HUGGETT AND NIXON[4] in this country and SAIFER AND GERSTENFELD[5] in America, introduced methods of glucose estimation based on the above principle, using *o*-dianisidine as oxygen acceptor. Both methods involved incubation at 37° for a minimum of 30 min.

MIDDLETON AND GRIFFITHS[6] introduced a method in which *o*-tolidine replaced *o*-dianisidine. It has the great advantage of rapidity, developing a blue colour at room temperature, and incubation is unnecessary. However, the reagent is unstable and a troublesome crystalline deposit is formed which must be removed by filtration before use.

It is intended to describe a modification of this method and some of its applications in clinical biochemistry.

METHOD

Reagents

The reagents used are, with the exception of the buffer, those described by MIDDLETON AND GRIFFITHS: (1) 0.9% NaCl. (2) 5% $ZnSO_4$. (3) 0.3 *N* NaOH. (4) 1% *o*-Tolidine in absolute alcohol. (5) "Fermcozyme" (Hughes & Hughes Ltd., London House, 35 Crutched Friars, London, E.C. 3), a stable liquid preparation containing 750 μg/ml, glucose oxidase. It appears to be stable almost indefinitely if refrigerated. (6) 0.15 *M* pH 5. Acetate buffer, made by adding approximately 30 parts acetic acid 0.15 *M* to 70 parts sodium acetate 0.15 *M* and adjusting final hydrogen ion concentration with a pH meter; (7) Stock peroxidase ("POD", C. F. Boehringer & Soehne, Mannheim, Germany), 20 mg% in acetate buffer. Lasts several months in the refrigerator. (8) Reagent: To about 80 ml of buffer add 0.5 ml of "Fermcozyme" and then 5 ml peroxidase solution. Mix and add 1.0 ml *o*-tolidine solution. Make up to 100 ml. Store in dark bottle in refrigerator. (9) Glucose standards: 2.5, 5.0, 7.5 and 10.0 mg glucose per 100 ml in saturated benzoic acid. These solutions are stable indefinitely at room temperature.

References p. 400

Procedure for blood

To 1.1 ml of saline, to which has been added shortly before use 0.4 ml $ZnSO_4$ solution and 0.4 ml 0.3 *N* NaOH, is added 0.1 ml of blood (if low values are expected substitute 1 ml saline and 0.2 ml blood). The mixture is centrifuged at 3000 r.p.m. for 4 min and separated as soon as possible, as in the presence of red cells and protein there is a slow but nevertheless significant disappearance of glucose. One ml of supernatant fluid is transferred to a test tube. Once separated the glucose content remains constant and can be determined at leisure within a period of 12 h. Into similar tubes place one ml of water for blank and one ml of 2.5, 5.0, 7.5 and 10.0 mg% glucose standards (equivalent to 50, 100, 150 and 200 mg % glucose in blood). 3 ml of reagent is added to each tube at half-minute intervals, mixed by gentle shaking for not more than 10 sec and the developed colour read on a spectrophotometer at 625 μ (or using E.E.L. filter O.R.I) exactly 10 min thereafter.

Anticoagulation and prevention of glycolysis

If for any reason it is not possible to deproteinise the blood immediately, coagulation may be prevented by addition of heparin 0.5 mg, potassium oxalate 5 mg, or E.D.T.A. 2 mg to each ml of blood, none of which interfere with the subsequent enzyme reaction. Unfortunately, it has not been found possible to prevent glycolysis with sodium fluoride since in quantities sufficient to prevent utilisation of glucose it very seriously interferes with the enzyme reaction (see Fig. 1). DENIS AND ALDRICH[7]

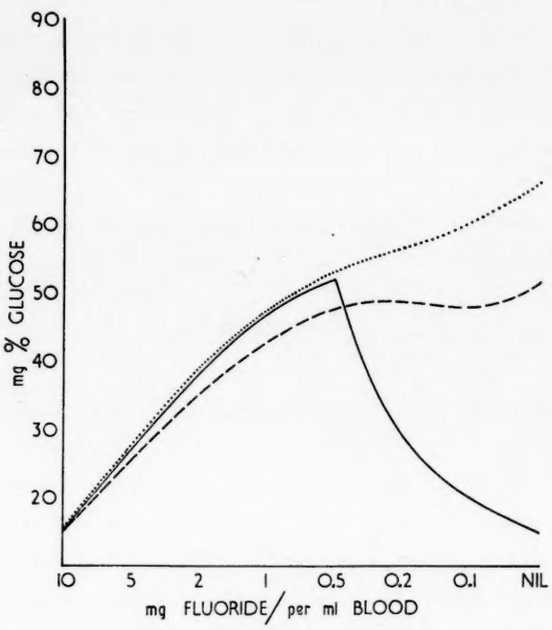


Fig. 1. Effect of sodium fluoride on the estimation of glucose by the glucose-oxidase method under varying conditions: ..... Blood deproteinised and separated immediately; — — — Blood deproteinised but not separated from precipitate for 4 h: ———— Blood incubated at 37° for 6 h before deproteinising and estimation.

suggested the use of formaldehyde 0.03 ml, 40% solution, per 5 ml blood for the preservation of blood glucose preparatory to estimation by the method of FOLIN AND WU[8]. We have found that 0.03 ml of 8% formaldehyde solution, per ml blood is

References p. 400

entirely adequate to prevent glycolysis for 24 h, at room temperature and does not interfere with the enzymic action. A three-fold increase in formaldehyde is without appreciable effect though larger amounts caused reduced recoveries.

Factors which influence the colorimeter procedure

Spectral curves. MIDDLETON AND GRIFFITHS used a Hilger Spekker with Kodak filter No. 570 (680 mμ). We have found that maximal optical densities were obtained at all concentrations with a wavelength of 625 mμ using Unicam SP 350 and SP 600 (see Fig. 2) and have accordingly used this wavelength in our experiments. Using

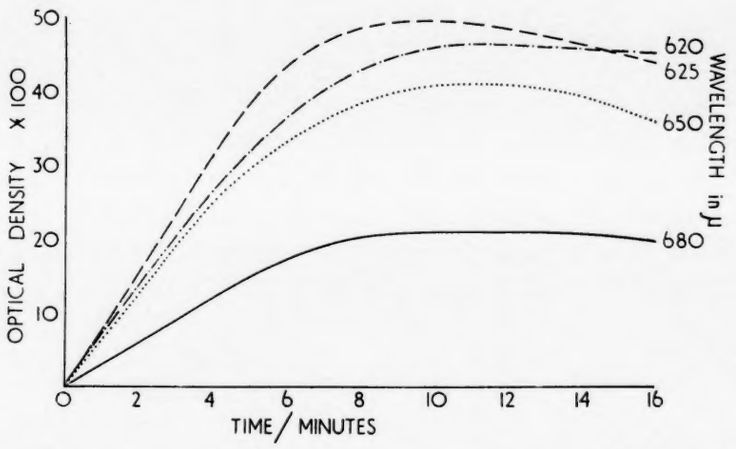


Fig. 2. Variations of optical density with time and wavelength (using Unicam S.P. 600: 1-cm cells) To 1 ml of 5 mg % glucose solution is added 3 ml reagent and the resultant colour read at the wavelengths and times shown.

the E.E.L. photometer we have found that filter O.R.I. gives results which comply with Beer's law.

Compliance with Beer's law. Perfect compliance was found for values of blood glucose between 10–200 mg % under conditions of the test. For values greater than 200 mg % 0.2 ml filtrate and 0.8 ml water should be used and for values below 30 mg a 1 in 10 dilution of blood should be used instead of the customary 1 in 20.

Temperature and length of incubation. Maximal colour development takes about 10 min at room temperature and declines slowly thereafter (see Fig. 2). External temperature within the limits of personal comfort has little effect on the rate of development, but if the reagent is used immediately on withdrawal from the refrigerator maximum colour development is delayed.

Reproducibility. Ten estimates were performed on a freshly drawn heparinised specimen of blood. Mean blood glucose level was 80.6 mg %. Standard deviation 1.7 mg % (*i.e.* S.D. = 2.1%). Range 78.5–82.5.

Stability of reagent. The reagent is ready for use immediately it is constituted. It maintains its activity for at least 3 days if stored in a refrigerator. The intensity of colour developed with any one standard remains almost constant over that period (over even longer periods it produces only small rises in colour intensity, *i.e.* 5%). Although intensity of colour is almost constant from one batch of reagent to another, enabling glucose levels to be read directly from a standard graph, it is recommended for accurate work that standards are put up with each run of estimations.

Recovery of added glucose is complete. (See Table I).

References p. 400

TABLE I

RECOVERY OF ADDED GLUCOSE FROM WHOLE BLOOD

Glucose added mg	*Found mg per 100 ml blood*	*Recovered mg*
Nil	55	Nil
50	105	50
25	80	25
20	76	21
15	70	15
10	64	9
5	60	5

Comparison with other methods. MIDDLETON AND GRIFFITHS[6] compared isolated levels of glucose as determined by glucose oxidase and by methods devised by MACLEAN[9] and ASATOOR AND KING[10]. We have determined glucose levels simultaneously by glucose oxide methods and that of FOLIN AND WU[11]. Seven estimations were made on each of 9 patients during the conduction of insulin tolerance tests

TABLE II

SIMULTANEOUS BLOOD SUGAR LEVELS AFTER INSULIN BY THE GLUCOSE-OXIDASE AND THE FOLIN AND WU METHODS

Insulin 0.1 units/kg body weight intravenously time (min)	*Folin and Wu*	*Glucose-oxidase*	*Mean difference*
Fasting	89.0	58.0	31
20	51.5	22.0	30
30	48.0	20.0	28
45	59.5	36.0	23.5
60	71.0	44.0	27
90	83.0	58.0	25
120	81.0	63.0	18

Results in mg glucose/100 ml.

(FRAZER *et al.*[12] the results of which were normal in all cases. The mean results are expressed in Table II. Our findings for normal fasting levels are similar to those of MIDDLETON AND GRIFFITHS, *i.e.* 50–90 mg %.

Procedure for C.S.F.

No inhibitory substances towards enzymatic reaction have been demonstrated in normal or pathological cerebrospinal fluid. As the fluid or its supernatant is almost invariably colourless, the method is simplified.

To 0.9 ml distilled water in a test tube is added 0.1 ml C.S.F.

3 ml of reagent is added and the colour developed is measured at 10 min. One hundred consecutive glucose analyses were made on cerebrospinal fluid obtained from patients, none of whom showed any fluid abnormality. The mean value obtained was 61.5 mg % S.D. 6.4 mg. The mean of 30 similar analyses by method of FOLIN AND WU was 67 mg % S.D. 9.5 mg.

References p. 400

Procedure for urine

The presence of large amounts of inhibitory substances, of which ascorbic acid (COMER[13]) and uric acid [3] are the best known, made direct estimation of the urinary glucose by this method impossible. TELLER recommended the removal of these inhibitors by the use of activated charcoal.

Method. 1 g of acid washed activated charcoal is added to a 20-ml aliquot of urine, thoroughly mixed for 10 min on a Kahn shaker and filtered through a No. 50 Whatman paper. A 1 in 10 or suitable dilution—giving a final glucose concentration of 0.5–10 mg %—is made using acetate buffer pH 5 as dilutant. To 1 ml of the diluted filtrate is added 3 ml of reagent and the colour developed is read at 10 min, as for blood.

Effect of pH. It is essential to use acid washed charcoal to remove all colouring and inhibitory substances from the urine. The resultant filtrate, if used undiluted has a pH sufficiently low to affect the buffering capacity of the reagent in the amounts used, giving incomplete recoveries of added glucose. Using a 1 in 10 dilution recoveries are almost complete, as can be seen in Table III.

TABLE III

RECOVERY FROM URINE OF ADDED GLUCOSE

Added mg	*Found* mg/100 ml urine	*Recovered* mg
Nil	6.5	Nil
12.5	17.5	11.0
25.0	26.0	19.5
50.0	52.0	45.5

DISCUSSION

The very large number of methods devised, and in current use, for the determination of blood glucose levels suggests the unsatisfactory nature of each. The majority depend on the reduction of either copper or ferricyanide compounds. It was early realised that these methods were of necessity non-specific, giving an index solely of total reducing activity of deproteinised blood.

With so large a number of methods of "sugar" estimation available there is an almost equally great number of "normal" fasting values, *e.g.* 90–120 (FOLIN AND WU[8]), 70–100 (KING AND WOOTTON[14]). Using the various glucose-oxidase methods, however, results are comparable, the normal fasting level for whole blood being 50–90 mg % (MIDDLETON AND GRIFFITHS[6]). SAIFER AND GERSTENFELD[5] found a mean plasma level of 90.9 mg $\pm$ S.D. 7.9 mg in 33 normal persons. For routine, as well as research laboratory work, the simple, quick and accurate method of MIDDLETON AND GRIFFITHS is ideal except in so far as the reagent is unstable. We have shown by substituting acetate for phosphate buffer that stability can be achieved.

In the past, attempts have been made to obtain true blood glucose levels by subtracting certain values—varying for each method—from the observed sugar levels. In the main these have been inadequate. In common with SAIFER AND GERSTENFELD we have not found that saccharoids bear any quantitative relationship to true glucose

References p. 400

levels. These authors found in addition that in their group of diabetic patients the non-glucose reducing substances are markedly elevated—not proportionately to the glucose content. This would seem to be related to the finding (see Table II) that insulin, in addition to reducing glucose levels in the blood, also causes, over a more prolonged period of time, a fall in the saccharoid fraction. In diabetes presumably the functional or true insulin deficiency permits an excess accumulation of these substances, the nature of which has not been determined.

FASHENA AND STIFF[15] believe that the saccharoid fraction in the blood of normal persons can be accounted for almost completely by the amounts of glutathione and glucuronic acid present. The possibility that the mono-phosphates of glucose may contribute must be borne in mind since these substances though not a substrate for glucose-oxidase are so for yeast and react with oxidising agents. These substances would seem a priori to be more likely influenced by insulin than the former.

Results in which there are so many unknown variables cannot justifiably be compared; when to this we add the difficulties produced by the use of different "blood sugar" methods, it is apparent that a strong case exists for abandoning the older non-specific methods of sugar estimation for one or other of the newer enzyme procedures.

SUMMARY

1. A simple, accurate, rapid method of determining glucose specifically in blood, C.S.F., and urine, using glucose-oxidase and peroxidase is described.

2. A comparison between glucose and non-glucose reducing fractions before, during and after insulin administration is made, in which it is shown that non-glucose reducing substances in blood are diminished by insulin over a prolonged period.

3. From the data presented it is suggested that the older non-specific methods of glucose estimation should be replaced by one of the newer enzyme methods.

REFERENCES

1 E. R. FROESCH AND E. A. RENOLD, *Diabetes*, 5 (1956) 1.
2 A. S. KESTON, *Abstr. of Papers 129th Meeting A.C.S., Dallas, Texas, April 1956*, p. 31c.
3 J. D. TELLER, *Abstr. of Papers 130th Meeting A.C.S., Atlantic City, N.J., Sept. 1956*, p. 69c.
4 A. St. G. HUGGETT AND D. A. NIXON, *Lancet*, 2 (1957) 368.
5 A. SAIFER AND S. GERSTENFELD, *J. Lab. Clin. Med.*, 51 (1958) 448.
6 J. E. MIDDLETON AND W. J. GRIFFITHS, *Brit. Med. J.*, 2 (1957) 1525.
7 W. DENIS AND M. ALDRICH, *J. Biol. Chem.*, 44 (1920) 203.
8 O. FOLIN AND M. WU, *J. Biol. Chem.*, 41 (1920) 367.
9 H. MACLEAN, *J. Physiol.*, 50 (1915) 168.
10 A. M. ASATOOR AND E. J. KING, *Biochem. J.*, 56 (1954) xliv.
11 G. A. HARRISON, *Chemical Methods in Clinical Medicine*, Churchill, London, 1957.
12 R. W. FRAZER, F. ALBRIGHT AND P. H. SMITH *J. Clin. Endocrinol.*, 1 (1941) 297.
13 J. P. COMER, *Anal. Chem.*, 28 (1956) 1748.
14 E. J. KING AND I. O. P. WOOTTON, *Micro-Analysis in Medical Biochemistry*, Churchill, London, 1957.
15 G. J. FASHENA AND H. A. STIFF *J. Biol. Chem.*, 137 (1941) 21.

Received October 13th, 1958

PHENOLSTÉROIDES URINAIRES

II. APPLICATIONS CLINIQUES

M. F. JAYLE, R. SCHOLLER ET F. VEYRIN-FORRER

Laboratoire d'Hormonologie de l'École de Puériculture, Faculté de Médecine, Paris (France)

Afin de dégager les perspectives qu'apporte le dosage des phénolstéroïdes urinaires[1] à l'exploration fonctionnelle des gonades et du placenta, nous allons présenter les premiers résultats que nous avons obtenus au cours de son application à différents groupes de sujets.

1. Cycles menstruels

(a) Cycles normaux. Au cours des publications antérieures[2-5], réalisées avec des méthodes de dosage des phénolstéroïdes moins spécifiques, nous avons montré que le tracé d'excrétion de ces métabolites au cours du cycle menstruel était biphasique. Partant d'un niveau bas au cours des dix premiers jours, il s'élève progressivement, présente un pic aigu au moment de l'ovulation; celui-ci est suivi d'une brusque descente et, après quelques jours, le tracé dessine un plateau qui précède la chute prémenstruelle.

Dans la présente étude nous avons dosé chez 113 femmes les phénolstéroïdes par le procédé que nous avons exposé précédemment[1]. Le prélèvement des urines a été réalisé dans tous les cas le 5ème jour du plateau thermique prémenstruel.

La première figure donne les résultats obtenus chez 55 femmes de 20 à 40 ans, dont la régularité des cycles, le plateau thermique, la menstruation, le poids étaient

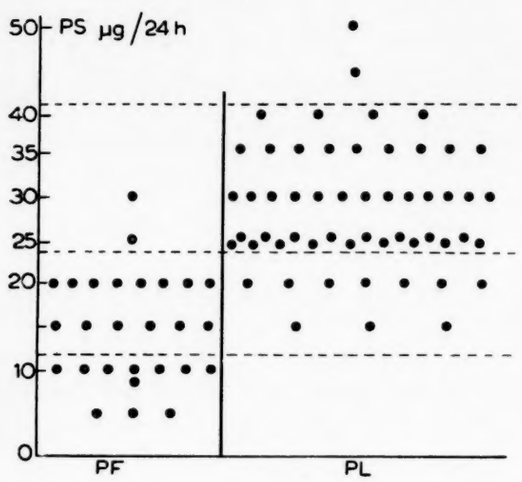


Fig. 1. Elimination des phénolstéroïdes au cours de 55 cycles normaux.
PF = Phases folliculaires (7e au 9e jours);
PL = Phases lutéales (5e ou 6e jour du plateau thermique prémenstruel).

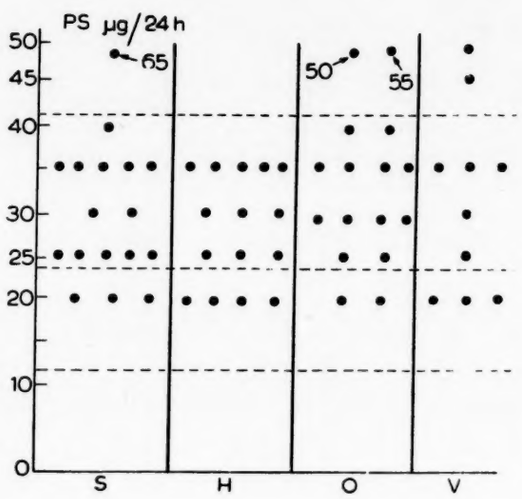


Fig. 2. Elimination des phénolstéroïdes au cours de cycles divers (Phases lutéales).
S = Spanioménorrhée; H = Hypoménorrhée; O = Obésité; V = Hirsutisme.

Bibliographie p. 410

normaux, et qui ne présentaient pas de signes de virilisme. Chez 27 de ces sujets un dosage a été fait en phase folliculaire, avant le 10ème jour du cycle menstruel. Ce groupe comprend des cas de stérilité et de fausses couches à répétition. Le taux moyen des phénolstéroïdes en phase lutéale est de 30 μg (15–50 μg) 91% des résultats étant compris entre 20 et 40 μg. En phase folliculaire, la moyenne est de 15 μg (5–30 μg), 93% des cas étant $\leqslant$ 20 μg.

(b) Cycles divers. La Fig. 2 donne les résultats en phase lutéale obtenus dans 17 cas de spanioménorrhée, 15 cas d'hypoménorrhée, 16 cas d'obésité et 10 cas d'hirsutisme. Les moyennes respectives de ces 4 groupes sont égales à: 31, 28, 33 et 31 μg. Le nombre des sujets est insuffisant dans chaque groupe pour permettre une étude statistique, mais la répartition des résultats ne paraît pas présenter de différences significatives avec les sujets normaux. Aussi les avons-nous utilisés en totalité pour construire la courbe de fréquence en phase lutéale, au 5e ou 6e jour du plateau thermique prémenstruel. La moyenne générale est de 30 μg ($\sigma = 7.8$); 91% des résultats sont compris entre 20 et 40 μg et 75% entre 25 et 40 μg. La courbe de fréquence, sans avoir une forme gaussienne, présente une symétrie satisfaisante.

On peut tirer de l'ensemble de ces données les conclusions suivantes: au 5ème jour du plateau thermique prémenstruel, l'activité œstrogène du corps jaune, mesurée

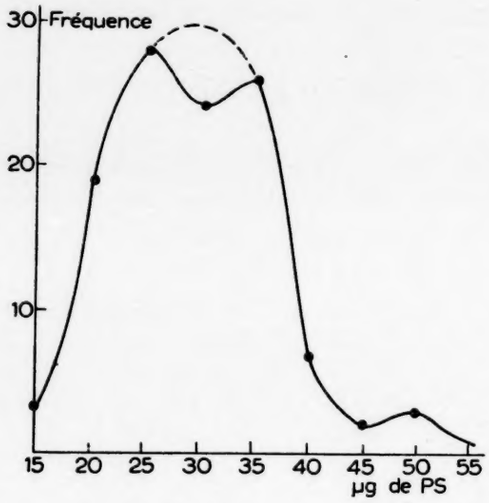


Fig. 3. Courbe de fréquence de l'élimination des phénolstéroïdes au cours de la phase lutéale de 113 cycles menstruels (5e ou 6e jour du plateau thermique prémenstruel).

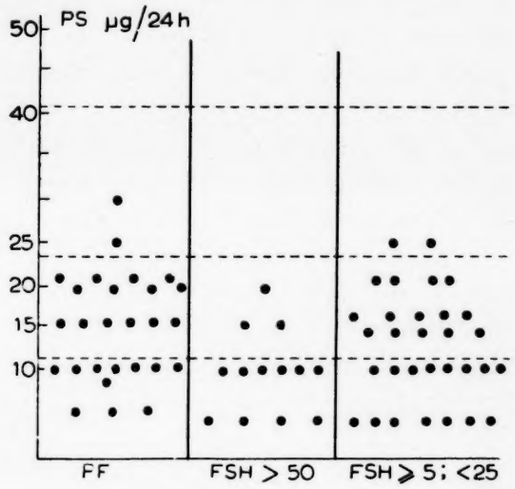


Fig. 4. Élimination des phénolstéroïdes au cours de 27 phases folliculaires normales (PF) et au cours d'aménorrhées hypergonadotropiques et hypothalamiques.

par le taux des phénolstéroïdes urinaires par la méthode décrite, est une constante biologique correspondant à 30 $\pm$ 10 μg. Il ne paraît pas y avoir de différences significatives de l'activité œstrogène du corps jaune, entre les sujets normaux et des sujets présentant de l'hypoménorhée, une spanioménorrhée, de l'hirsutisme ou une obésité.

2. Aménorrhées

La Fig. 4 groupe 13 aménorrhées avec un taux de FSH* > 50 U.S. comprenant

* Follicle stimulating hormone.

Bibliographie p. 410

principalement des castrations et des ménopauses et 31 aménorrhées avec un taux de FSH $> 5 < 25$ U.S.

Les 13 aménorrhées hypergonadotropiques ont un taux moyen de phénolstéroïdes égal à 10 μg (5 à 20) dont 12 ont un taux $\leqslant 15$ μg. La valeur moyenne des phénolstéroïdes du second groupe est de 13 μg (5–25); 6 cas seulement ont des taux > 15 μg (Fig. 4).

On peut tirer les conclusions suivantes. Les taux des phénolstéroïdes au cours des aménorrhées hypergonadotropiques et hypothalamiques sont semblables dans la majorité des cas; ils ne diffèrent pas des valeurs constatées avant le 10ème jour des cycles menstruels normaux; cependant, le fait de trouver au cours d'une aménorrhée un taux de phénolstéroïdes $\geqslant 20$ μg témoigne, avec une bonne probabilité, de l'activité œstrogène de l'ovaire.

3. *Hommes et enfants*

Le taux moyen trouvé chez 23 hommes est de 17 μg (10–30 μg), 95% des résultats étant compris entre 10 et 25 μg. Ces valeurs sont voisines de celles que l'on trouve avant le 10ème jour du cycle menstruel normal (Fig. 5).

Il existe une différence significative entre le taux moyen des enfants impubères, égal à 4 μg (0–7), et celui des enfants pubères, qui est de 12 μg (5–15 μg).

4. *Ménométrorragies*

Nous avons discuté ailleurs[7] la pathogénie hormonale des ménométrorragies en nous basant sur les déterminations des phénolstéroïdes, du pregnandiol et des 17-cétostéroïdes. Nous ne mentionnerons ici que les dosages de phénolstéroïdes.

Sur la Fig. 6 nous avons groupé les résultats obtenus chez 56 sujets présentant des ménorragies ou des métrorragies; nous les avons répartis en deux catégories, selon que les sujets étudiés présentaient ou non un cycle menstruel avec plateau thermique. Dans 38 cas les dosages ont été faits au 5ème jour du plateau thermique et dans 18 cas seulement en phase hypothermique.

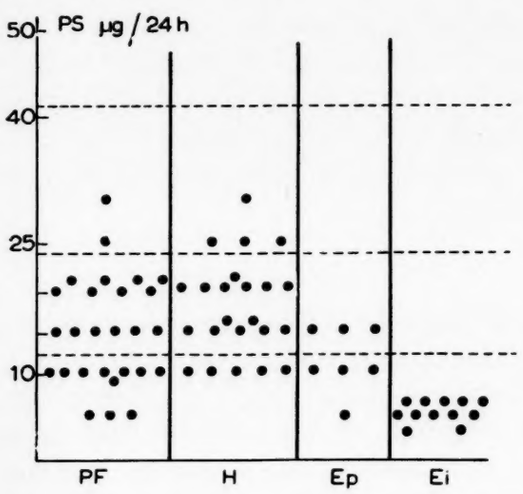


Fig. 5. Élimination des phénolstéroïdes chez 23 hommes et 19 enfants. PF = Phases folliculaires de cycles normaux; H = Hommes; E_i = Enfants impubères; E_p = Enfants pubères.

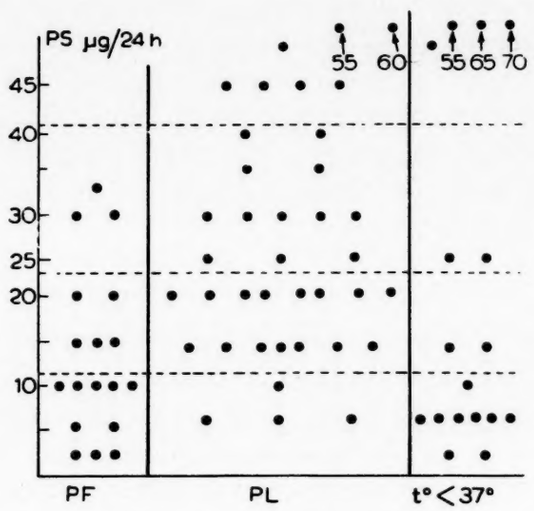


Fig. 6. Élimination des phénolstéroïdes au cours de ménométrorragies. PF = Phases folliculaires; PL = Phases lutéales; $t < 37°$ = Sujets ne présentant pas de plateau thermique $> 37°$.

Bibliographie p. 410

Dans la première catégorie, comptant 38 observations, nous trouvons en phase lutéale 20 résultats normaux (de 20 à 40 μg), 11 insuffisants et 7 résultats augmentés ($\geqslant$ 45 μg) ; parmi ces derniers trois seulement sont supérieurs à 50 μg. Dans les résultats normaux nous trouvons 8 fois sur 20 des taux de 20 μg, ce que nous considérons comme étant insuffisant au milieu d'une phase lutéale ; cela met l'accent sur la prédominance de l'insuffisance œstrogénique du corps jaune dans le déterminisme des ménométrorragies.

Dans la seconde catégorie comprenant 17 observations chez des femmes ne présentant pas de décalage thermique cyclique, nous trouvons 4 résultats $\geqslant$ 50 μg, ce qui porte à 11 sur 55 le nombre de patientes présentant un excès de phénolstéroïdes urinaires.

Chez 11 sujets de cette catégorie, les taux de phénolstéroïdes sont $\leqslant$ 15 μg.

On peut en tirer les conclusions suivantes : l'hyperfolliculinie, se traduisant par un taux de phénolstéroïdes $\geqslant$ 50 μg/24 h, ne se rencontre que dans 20% des cas de ménométrorragie. 30 sujets, soit 54%, présentent des taux de phénolstéroïdes $\leqslant$ 20 μg. L'insuffisance folliculinique parait donc jouer un rôle plus important que l'hyperfolliculinie dans le déclenchement des ménorragies et des métrorragies.

5. Hyperfolliculinie

L'ensemble de ces résultats nous amène à reconsidérer la notion d'hyperfolliculinie en endocrinologie génitale. Avec une technique de dosage de la folliculine, moins spécifique que celle dont nous disposons actuellement, nous avions déjà montré avec GELLER[8] que l'hyperfolliculinie était exceptionnelle dans le syndrome congestif prémenstruel. Sur 213 dosages de phénolstéroïdes pratiqués au cours de 113 cycles menstruels, normaux ou divers, et 55 cas de ménorragies et de métrorragies, nous relevons seulement 12 dosages $\geqslant$ 50 μg (5.6%), dont 7 cas dans les groupes des ménométrorragies. Chez les femmes présentant un cycle menstruel, avec ou sans ménométrorragies, tous les cas d'hyperfolliculinie ont été trouvés au milieu de la phase lutéale, l'excrétion en phase folliculaire étant toujours normale. Dans nos recherches antérieures, nous avions constaté que l'hyperfolliculinie en phase lutéale était fréquente dans la dégénérescence kystique de l'ovaire[9]. On peut conclure de ces données que l'hyperfolliculinie est rare et qu'elle se rencontre principalement dans certaines formes de ménométrorragies et de kyste folliculinique de l'ovaire. Comme nous l'avons mentionné dans une publication récente[7], c'est l'insuffisance lutéale, plus souvent que l'hyperfolliculinie, qui doit être rendue responsable de l'hyperplasie glandulokystique de l'endomètre.

6. Phénolstéroïdes d'origine surrénale

Normalement la cortico-surrénale ne produit, dans les deux sexes, que de faibles quantités de phénolstéroïdes. On peut en apprécier l'ordre de grandeur par le taux moyen de 10 μg trouvé chez les femmes castrées ou ménopausées. L'administration à ces sujets de 20.000 ou de 30.000 unités de gonadotropines chorioniques n'est suivie d'aucune augmentation du taux de ces métabolites[9]. Il en est de même des hommes castrés ou eunuchoïdes[10]. La perfusion endoveineuse unique de 25 U.I. d'ACTH ne modifie pas le taux d'excrétion des phénolstéroïdes d'une façon significative[11].

Dans la maladie de Cushing, le taux des phénolstéroïdes n'est pas augmenté ; dans le cancer de la corticosurrénale il est normal dans la majorité des formes virili-

Bibliographie p. 410

santes de cette affection, mais nous l'avons trouvé augmenté, parfois même très fortement, dans certaines formes féminisantes qui sont beaucoup plus rares que les précédentes. Dans l'hyperplasie congénitale de la corticosurrénale, le taux des phénolstéroïdes est toujours augmenté.

Tels sont les résultats que nous avons constatés dans l'ensemble de nos observations de 1950 à 1957. Les dosages des phénolstéroïdes n'ayant pas été pratiqués chez ces patients avec notre technique récente, nous ne mentionnerons pas ici les résultats.

7. Influence des gonadotropines chorioniques sur l'excrétion des phénolstéroïdes chez la femme en phase lutéale

Au cours de recherches antérieures[9-12], nous avons montré que l'administration de 10.000 U.I. de gonadotropines chorioniques au 3ème et au 5ème jour du plateau thermique prémenstruel, transformait le corps jaune menstruel en un corps jaune pseudo-gestatif; l'excrétion du pregnandiol et des phénolstéroïdes est maximum entre les 6ème et 8ème jours après la premiére injection. Nous avons groupé sur la Fig 7 les résultats les plus récents que nous avons obtenus en utilisant notre nouvelle technique de dosage des phénolstéroïdes, chez des femmes dont la majeure partie présentait soit une stérilité primaire ou secondaire, soit des fausses couches à répétition. Nous avons distingué deux catégories selon que le taux des phénolstéroïdes en phase lutéale témoin était normal ou notoirement insuffisant. Dans 23 cas, le taux en phase lutéale témoin était compris entre 20 et 40 μg avec une moyenne de 26 μg. Après l'épreuve, la valeur moyenne s'élève à 65 μg; cinq fois le taux des phénolstéroïdes demeure < 50 μg; dans 3 de ces cas il n'était que de 20 μg en phase lutéale témoin (Fig. 7).

Chez 18 sujets, le taux des phénolstéroïdes était $\leqslant 15$ μg, soit nettement insuffisant, en phase lutéale témoin. Au 7ème jour de l'épreuve pratiquée au cycle suivant, la valeur moyenne est de 48 μg; 12 fois le taux des phénolstéroïdes demeure < 50 μg. On peut tirer de ces résultats les conclusions suivantes.

Lorsqu'en phase lutéale témoin, le taux des phénolstéroïdes est $\geqslant 20$ μg, on observe une augmentation des phénolstéroïdes à un taux $\geqslant 50$ μg par 24 h dans 78% des cas; lorsque le taux des phénolstéroïdes en phase lutéale témoin est insuffisant, le taux d'excrétion des phénolstéroïdes après l'épreuve demeure dans 67% des cas inférieur à 50 μg. Il existe donc, dans la majorité des cas, une bonne corrélation entre l'activité œstrogène du corps jaune menstruel et celle du corps jaune pseudogestatif.

Cette épreuve aux gonadotropines chorioniques est cependant nécessaire pour apprécier la valeur fonctionnelle de la thèque interne et pour faire la distinction entre l'insuffisance folliculinique du corps jaune, dont l'origine est ovarienne, et celle dont l'origine est probablement hypophysaire. Dans un travail antérieur[7], nous avons montré la fréquence des insuffisances folliculiniques du corps jaune chez des patientes présentant des stérilités ou des fausses couches à répétition. Nous avons également montré qu'il n'existait aucune corrélation entre les valeurs du pregnandiol et celles des phénolstéroïdes avant comme après administration de gonadotropines chorioniques.

8. Influence des gonadotropines chorioniques sur l'excrétion des phénolstéroïdes chez l'homme

La Fig. 8 donne les résultats obtenus chez 16 hommes normaux de 20 à 50 ans, avant et après administration de gonadotropines chorioniques en deux ou trois jours

Bibliographie p. 410

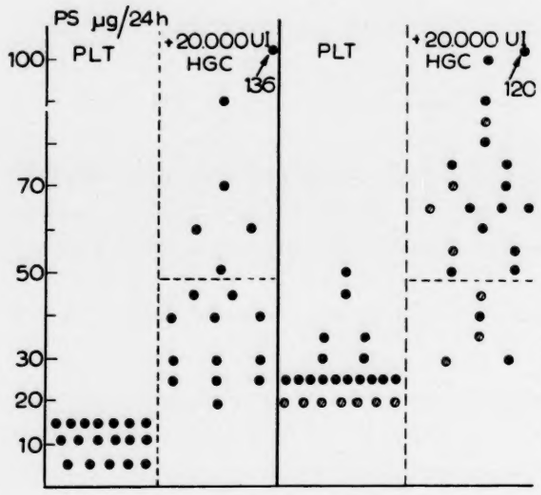


Fig. 7. Taux des phénolstéroïdes (PS) urinaires avant et après administration de 20.000 U.I. de HGC. en phase lutéale. A gauche, taux de PS $\leqslant$ 15 μg en phase lutéale témoin (PLT). A droite, taux de PS $\geqslant$ 20 μg, $\leqslant$ 40 μg en phase lutéale témoin (PLT). Cercles hachurés = 20 μg en PLT.

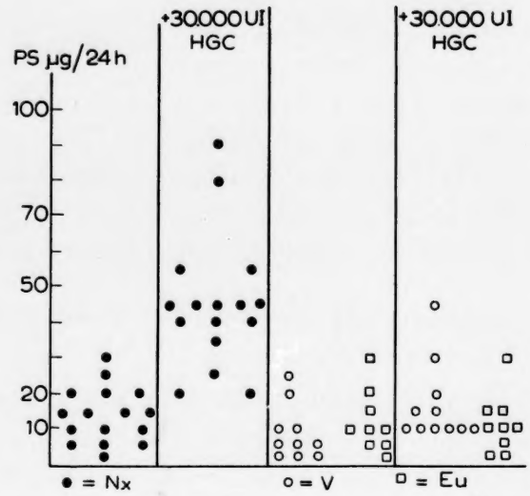


Fig. 8. Élimination des phénolstéroïdes chez des hommes normaux (Nx), des vieillards (V) et des eunuchoïdes (Eu) avant et après administration de 20,000 ou de 30,000 U.I. de gonadotropines chorioniques (HGC).

consécutifs. Les urines sont rejetées au moment de la seconde ou de la troisième injection, selon que l'on administre 20.000 ou 30.000 U.I. de gonadotropines chorioniques; le dosage est pratiqué sur les urines des 24 h suivantes. On constate une augmentation significative des phénolstéroïdes, dont la valeur moyenne, de 14 μg (0–30) avant l'épreuve, atteint 45 μg (20–90) après celle-ci. Nous estimons que ce test a une valeur qualitative et spécifique de l'activité endocrinienne des cellules de Leydig; il ne paraît pas avoir le caractère quantitatif que nous avons signalé au cours de la phase lutéale du cycle menstruel.

Chez les hommes castrés, chez les eunuchoïdes et chez les vieillards, on ne constate aucune augmentation des phénolstéroïdes sous l'action des gonadotropines chorioniques.

9. Excrétion des phénolstéroïdes au cours de la grossesse

Le Tableau I donne les valeurs moyennes des phénolstéroïdes de la 5ème à la 41ème semaine après les dernières règles, au cours des grossesses ayant évolué sans présenter des troubles graves. Nous estimons que les variations physiologiques du taux des phénolstéroïdes urinaires vont de 60% des valeurs moyennes normales à 150%. De 60 à 40% des valeurs moyennes normales se situe la zone de l'insuffisance folliculinique modérée. Au-dessous de 40%, il existe une insuffisance folliculinique sévère dont nous avons indiqué la valeur séméiologique dans des recherches antérieures[13].

Le Tableau II donne une idée de la constance du taux moyen d'excrétion des phénolstéroïdes au cours de grossesses ayant évolué sans troubles sérieux. Aux différentes époques de grossesses âgées de 5 à 14 semaines, nous avons établi les moyennes sur trois populations différentes. La comparaison de ces nombres donne une idée de la constance physiologique de l'activité œstrogène du corps jaune gestatif et du placenta.

Bibliographie p. 410

TABLEAU I

ÉLIMINATION MOYENNE DES PHÉNOLSTEROÏDES AU COURS DE LA GROSSESSE

(μg/24 heures)

Semaines	*PS*	*Semaines*	*PS*
5–6	115	23–24	7.600
7–8	180	25–26	9.350
9–10	210	27–28	10.100
11–12	350	29–30	11.300
13–14	660	31–32	12.300
15	1.450	33–34	16.100
16	2.300	35–36	17.450
17–18	3.300	37–38	21.400
19–20	4.500	39–40	22.300
21–22	6.000		

PS = phénolstéroïdes

TABLEAU II

ÉLIMINATION DES PHÉNOLSTEROÏDES À PARTIR DU DEUXIÈME TRIMESTRE DE LA GESTATION

(μg/24 h)

Semaines	*15–16*	*17–18*	*19–20*	*21–22*	*23–24*
1er groupe	1.050	1.940	2.400	3.600	3.700
2ème ,,	1.260	1.815	2.660	3.580	4.485
3ème ,,	1.275	1.840	2.500	3.350	4.230
Semaines	*25–26*	*27–28*	*29–30*	*31–32*	*33–34*
1er groupe	5.100	5.360	5.500	6.800	8.600
2ème ,,	5.150	6.410	5.560	7.770	9.520
3ème ,,	5.200	5.600	6.275	8.930	
Semaines	*35–36*	*37–38*	*39–40*	*41–42*	
1er groupe	10.150	12.850	11.850	—	
2ème ,,	11.770	13.670	15.140	13.460	
3ème ,,	9.685	11.900	12.400	14.360	

Au début de la grossesse, le dosage des phénolstéroïdes peut être utilisé valablement pour le diagnostic de la gestation. En effet, d'après une statistique réalisée avec ROUSSANGE[14], portant sur des dosages pratiqués entre la 5ème et la 10ème semaine après les dernières règles, 96.5% des résultats obtenus étaient supérieurs ou égaux à 50 μg. Dans 3.5 % des grossesses hypofolliculiniques, le dosage du pregnandiol était soit dans la zone gestative (> 8 mg), soit dans l'aire du corps jaune fonctionnel d'un cycle menstruel (4 à 8 mg). Chez plusieurs patientes devenues enceintes, des dosages de phénolstéroïdes ont été pratiqués avant la date présumée des prochaines règles et ont montré une augmentation significative du pregnandiol et des phénolstéroïdes, alors que le diagnostic biologique sur lapine était négatif avec 3 ml de sérum sanguin.

Bibliographie p. 410

Si nous comparons l'excrétion des phénolstéroïdes au début de la grossesse, avec celle des aménorrhées hypothalamiques de la Fig. 4, nous voyons la valeur diagnostique de ce test. Au milieu de la phase lutéale des 113 cycles menstruels étudiés plus haut (Fig. 3), nous n'avons trouvé que 5 fois des taux de phénolstéroïdes $> 50\ \mu g$, soit dans 4.5% des cas.

Dans la population de grossesses à laquelle nous nous référons, il y a une proportion importante de patientes ayant présenté de nombreux accidents antérieurs, ou encore se signalant par des contractions douloureuses à l'époque des analyses. Cela laisse supposer que, sur une population de grossesses normales, la valeur diagnostique de cette méthode serait encore plus grande. Nous pouvons tirer de cette étude et de nos recherches antérieures les conclusions suivantes:

(a) Au début de la grossesse, le diagnostic de l'état gravidique fondé sur le dosage des phénolstéroïdes a une valeur égale au diagnostic biologique sur lapine;

(b) Un taux de phénolstéroïdes $< 25\ \mu g$ associé à un taux de pregnandiol $<$ 2 mg permet d'affirmer l'absence ou l'interruption de la grossesse;

(c) l'insuffisance folliculinique sévère présente une grande valeur séméïologique aux différentes époques de la gestation.

10. Exploration fonctionnelle des gonades

(a) Exploration de la fonction lutéale. Nous avons standardisé l'exploration fonctionnelle de l'ovaire conformément au schéma de la Fig. 9.

La température est prise tous les matins; des frottis vaginaux sont effectués par la malade tous les 3 jours après la fin des règles jusqu'au début du cycle suivant. Dans certains cas, une biopsie est pratiquée entre le 8ème et le 10ème jour du plateau thermique prémenstruel; les urines de 24 heures sont collectées le 5ème ou le 6ème jour du plateau thermique prémenstruel; les dosages des phénolstéroïdes, du prégnandiol, des 17-cétostéroïdes et de la créatinine sont pratiqués.

Par les méthodes que nous utilisons, les taux normaux au 5ème jour du plateau

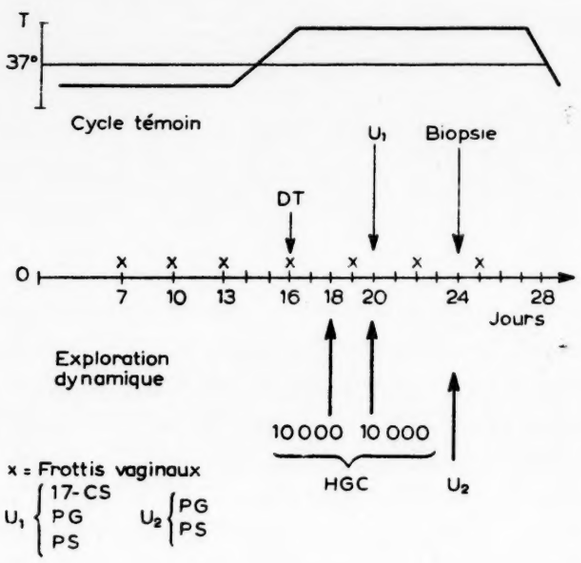


Fig. 9. Schéma d'exploration du cycle menstruel. U_1 = prélèvement urinaire en phase lutéale témoin. U_2 = prélèvement urinaire après 2 × 10,000 U.I. de HGC. DT = décalage thermique au-dessus de 37°.

Bibliographie p. 410

thermique sont compris entre 20 et 40 μg pour les phénolstéroïdes, entre 4 et 8 mg pour le pregnandiol et entre 5 et 13 mg pour les 17-cétostéroïdes. L'épreuve dynamique de la fonction lutéale consiste à administrer entre le 3ème et le 6ème jour du plateau thermique prémenstruel, 20.000 U.I. de gonadotropines chorioniques, en deux injections de 10.000 unités. Les urines sont recueillies le 7ème jour suivant la première injection.

Après administration de gonadotropines chorioniques, le taux du pregnandiol s'élève normalement entre 8 et 15 mg, celui des phénolstéroïdes entre 40 et 80 μg; l'épreuve est alors considérée comme étant normale. Dans l'insuffisance lutéale globale, les taux du pregnandiol et de la folliculine sont d'autant plus faibles que l'insuffisance est plus sévère. L'insuffisance lutéale peut être dissociée et, dans ce cas, seul le taux du pregnandiol ou celui de la folliculine demeure nettement insuffisant après l'épreuve.

(b) Exploration de l'ovaire au cours de l'aménorrhée. Les urines de 24 heures sont prélevées pendant deux jours consécutifs; sur le premier prélèvement, un dosage de FSH est pratiqué sur des souris impubères. On recherche 5, 25 et 50 U.S. Lorsque la réponse à 5 unités est négative, on recherche 3 unités sur un nouveau prélèvement d'urines. Sur le deuxième prélèvement urinaire, les dosages de la créatinine, des phénolstéroïdes et des 17-cétostéroïdes sont pratiqués.

En outre, plusieurs frottis vaginaux sont effectués et la courbe de la température matinale est établie pendant une assez longue période.

Lorsque le taux de FSH est supérieur à 50 U.S., il s'agit d'une ménopause précoce ou d'une agénésie ovarienne. S'il est inférieur à 25 unités et supérieur à 3 ou 5 unités, l'aménorrhée est probablement d'origine hypothalamique.

La constatation d'un taux de phénolstéroïdes supérieur à 20 μg est l'indice d'une activité œstrogène de l'ovaire. Lorsque le taux des phénolstéroïdes est inférieur à cette valeur et lorsque le taux de FSH est négatif à 50 U.S., nous administrons 30.000 U.I. de gonadotropines chorioniques en six injections de 5.000 unités*; les urines de 24 h sont prélevées le 7ème jour après la première injection. Les dosages de phénolstéroïdes, de pregnandiol et de créatinine sont effectués. Une augmentation significative de ces deux catégories de métabolites est considérée comme l'indice d'une activité endocrinienne de l'ovaire. Lorsque la réponse est négative, il existe une probabilité de l'origine ovarienne de l'aménorrhée, même lorsque le taux de FSH est normal.

(c) Exploration de la fonction endocrinienne du testicule. On administre, pendant trois jours consécutifs, 10.000 U.I. de gonadotropines chorioniques. Les urines de 24 h sont prélevées avant l'épreuve; les urines sont rejetées au moment de la troisième injection et on prélève les 24 h suivantes. Sur les deux prélèvements urinaires, on pratique les dosages de 17-cétostéroïdes, de phénolstéroïdes et de créatinine. Nous estimons que la réponse testiculaire est satisfaisante lorsque, pour des taux de créatinine sensiblement identiques, l'excrétion des 17-cétostéroïdes s'est élevée de 50 à 100% au-dessus du niveau de départ et lorsque le taux des phénolstéroïdes dépasse 30 μg.

---

* Il est préférable de faire précéder cette épreuve de deux cycles artificiels et de commencer les injections de HGC le 20 ième jour après le saignement.

Bibliographie p. 410

RÉSUMÉ

Nous avons présenté dans ce travail les taux des phénolstéroïdes au cours des deux phases de cycles menstruels normaux et pathologiques, au cours d'aménorrhées, de ménométrorragies, à différents stades de la grossesse, chez l'homme, chez l'enfant pubère et impubère. Nous avons étudié l'excrétion de ces métabolites, avant et après administration de 20.000 ou 30.000 U.I. de gonadotropines chorioniques, au milieu de la phase lutéale ou chez l'homme. Nous avons montré la valeur séméiologique de cette détermination au cours du cycle menstruel, de l'aménorrhée et de la grossesse. Nous avons proposé des méthodes standardisées en vue de l'exploration endocrinienne de la fonction lutéale, de l'aménorrhée et du testicule.

SUMMARY

PHENOLIC STEROIDS IN THE URINE

II. CLINICAL APPLICATIONS

The content of phenolic steroids in the urine was determined: during the two phases of normal and pathological menstrual cycles, in cases of amenorrhoea and menometrorrhagia, in various stages of pregnancy, in men, and in children before and during puberty. The excretion of these metabolites was studied before and after administration of 20,000 of 30,000 I.U. of chorionic gonadotropins, in women in the middle of the luteal phase or in men. The authors show that their method has symptomatological value for determinations during the course of the menstrual cycle, in cases of amenorrhoea and in pregnancy. Standard methods are proposed for endocrinological investigations concerning the luteal function, amenorrhoea, and the testicle.

BIBLIOGRAPHIE

[1] M. F. JAYLE, R. SCHOLLER, M. HERON ET S. METAY, *Clin. Chim. Acta*, 4 (1959) 276.
[2] M. F. JAYLE, O. CRÉPY ET O. JUDAS, *Bull. soc. chim. biol.*, 31 (1949) 1592.
[3] M. F. JAYLE, O. CRÉPY ET O. JUDAS, *Ann. endocrinol. (Paris)*, 11 (1950) 97.
[4] M. F. JAYLE, O. CRÉPY, B. SCHRAMM, S. VANDEL ET O. JUDAS, *Ann. endocrinol. (Paris)*, 11 (1950) 545.
[5] M. F. JAYLE ET O. CRÉPY, *Compt. rend. soc. biol.*, 145 (1951) 269.
[6] M. F. JAYLE ET O. CRÉPY, *Colloques de la fonction lutéale*, Vol. 1, p. 103, Paris, 1954.
[7] M. F. JAYLE ET F. VEYRIN-FORRER, *Journées gynécologiques franco-roumaines*, Paris, 1957.
[8] S. GELLER, Y. VALLIN ET M. F. JAYLE, *Rev. franç. d'études clin. biol.*, 1 (1956) 318.
[9] M. F. JAYLE, J. GUEGUEN, Y. VALLIN ET F. VEYRIN-FORRER, *Presse méd.*, 64 (1956) 85, 1965.
[10] M. F. JAYLE, R. SCHOLLER, G. GARRONE ET P. MOREL, *Colloques sur la fonction endocrine du testicule*, Paris, 1957.
[11] M. F. JAYLE, *Bruxelles méd.*, 36 (1956) 231.
[12] M. F. JAYLE, *Bull. soc. roy. belge gynécol. et obstét.*, 27 (1957) 59.
[13] M. F. JAYLE ET O. CRÉPY, *Gynécol. et obstét.*, 55 (1954) 1.
[14] G. ROUSSANGE, *Thèse Médecine*, Paris, 1957.
[15] O. CRÉPY, F. MESLIN ET M. F. JAYLE, *Bull. soc. chim. biol.*, 38 (1956) 505.

Reçu le 12 juin 1959

THE DETERMINATION IN URINE OF SOME METABOLITES OF TRYPTOPHAN—KYNURENINE, ANTHRANILIC ACID AND 3-HYDROXYANTHRANILIC ACID—AND REFERENCE TO THE PRESENCE OF *o*-AMINOPHENOL IN URINE

S. L. TOMPSETT

Department of Clinical Chemistry, University of Edinburgh (Great Britain)

The metabolites of tryptophan that so far have been identified in human urine exhibit quite marked differences in chemical structure and properties. Recently there has been an interest in the relationship of these metabolities to disease[1-3] and certain vitamin deficiencies[4]. It has been suggested that those aromatic substances containing an *ortho*-hydroxamic structure may have an association with malignant disease of the bladder and naturally an interest has been taken in those metabolites of tryptophan possessing this structure, *viz.* 3-hydroxyanthranilic acid and 3-hydroxykynurenine[5-8]. A marked increase in the urinary excretion of anthranilic acid (*o*-aminobenzoic acid) has been reported in congenital hypoplastic anaemia[9].

A great variety of procedures have been employed in the determination of tryptophan metabolites—paper chromatography[1-4], partition chromatography followed by ultraviolet spectrophotometry, ion-exchange resins to effect separations[11], etc. COPPINI[12] has written a very extensive review on the subject. Much further investigation appears to be necessary and in particular there appears to be a lack of suitable methods for the determination of substances with an *ortho*-hydroxamic structure.

The present investigation is limited to the determination of the following metabolites of tryptophan:

1. diazotisable aromatic amines—kynurenine and anthranilic acid,
2. *ortho*-hydroxyamines—3-hydroxyanthranilic acid.

o-Aminophenol is included although there is no conclusive evidence that it is wholly or partly derived from tryptophan.

The intention was to determine the total amount of a substance whether it was in the free or conjugated state. Prior to examination, therefore, 10 ml urine are placed in a test tube together with 1 ml 10 *N* hydrochloric acid and the whole placed in a boiling water bath for 1 h. This process does not appear to affect the parent substances.

PROCEDURES

Diazotisable aromatic substances

Colorimetric estimations are carried out essentially by the BRATTON AND MARSHALL technique[13] which involves diazotisation with nitrous acid in acid solution followed by coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride. Some degree of specificity may sometimes be achieved by allowing the coupling process to take place (1) in the presence of 0.2 *N* hydrochloric acid, (2) at pH 7.0 or (3) in the presence

References p. 419

of high concentrations of hydrochloric acid and sodium chloride. Investigations showed that such devices did not achieve the degree of specificity required and hence coupling is allowed to take place in the presence of 0.2 *N* hydrochloric acid. The coupling process is rapid (maximum in 15 min) in the case of those diazotised aromatic amines with a substituent in the *para* position but slow (maximum in 2 h) in the case of those with a substituent in the *ortho* position. This property has proved of considerable value in the examination of mixtures of these two groups of amines. *ortho*-Hydroxyamines react with nitrous acid in acid solution to produce a yellow colour but this product does not couple with *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Interference by this reaction is usually minimal but if necessary, corrections for the yellow colour may be applied.

Reagents. (1) Hydrochloric acid 10 *N*; (2) 0.1% (w/v) sodium nitrite, freshly prepared; (3) 0.5% (w/v) ammonium sulphamate; (4) 0.1% (w/v) N-(l-naphthyl)-ethylenediamine dihydrochloride.

Kynurenine

Several methods have been suggested for the determination of kynurenine in urine[14–16]. Probably that proposed by ŠPAČEK[17] achieves most closely the degree of specificity required. In this method, distillation is carried out in the presence of sodium hydroxide, kynurenine being converted into *o*-aminoacetophenone which is recoverable in the distillate. ŠPAČEK assessed kynurenine from measurements made in the ultra-violet part of the spectrum but interferences were experienced from such substances as *p*-phenetidine if present. In the procedure described in this paper, the details of the distillation process as described by ŠPAČEK are adhered to but the content of amine in the distillate is determined by the BRATTON AND MARSHALL technique, coupling being allowed to proceed for 2 h since one is dealing with an amine with a substituent group in the *ortho* position. *p*-Phenetidine was found not to interfere. A control distillation is carried out at neutrality to determine the presence, if any, of non-specific volatile amines. The following are the details of the proposed procedure.

Method. The distillation process is carried out in an all glass apparatus: a 100-ml distillation flask attached to a watercooled condenser. Into the distillation flask are introduced 10 ml of acid-hydrolysed urine, 30 ml of water and 5 g of sodium hydroxide. The mixture is boiled until 20 ml of distillate have collected.

To 10 ml of distillate are added 0.2 ml of 10 *N* hydrochloric acid followed by 1 ml of 0.1% sodium nitrite. After standing 5 min at room temperature, 1 ml of 0.5% ammonium sulphamate is added and after standing at room temperature for a further 5 min. 1 ml of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride is added. After standing at room temperature for 2 h, to allow maximum colour development, absorptions are read against a blank at 540 mμ.

Standards containing 25, 50 and 100 μg of kynurenine should be set up att he same time, the complete procedure being used.

When kynurenine, 10 to 200 μg, was examined by the above procedure, the relationship between light absorption at 540 mμ and concentration was found to be linear.

Kynurenine, 25 to 200 μg, added to 10 ml of urine, could be determined with an accuracy of 89 to 98%.

Specificity. The following substances were examined by the above technique and

References p. 419

found not to produce any volatile diazotisable amine; *o*- and *p*-aminobenzoic acids, *o*- and *p*-aminophenols, tryptophan, indolylacetic acid, β-(3-indolyl)-propionic acid, phenacetin, *p*-phenetidine and 3-hydroxykynurenine. From indole, however, volatile diazotisable amines were produced. As a result, a control distillation is always carried out on acid-hydrolysed urine which has been neutralised to pH 7.4. Non-specific diazotisable and volatile amines were not detectable in normal urine but appreciable quantities were generally found in urine from cases of malignant disease of the bladder. The nature of these has not been determined but they are probably of putrefactive origin.

Aminobenzoic acids

Both *o*- and *p*-aminobenzoic acids occur in urine and both produce compounds of similar colour after diazotisation and coupling. LEMBERG *et al.*[18] effected a separation of the two coloured compounds by the use of alumina. In the present investigation it has been noted that (1) with *p*-aminobenzoic acid the maximum intensity of colour is reached 15 min after coupling and remains unchanged for at least 2 h, (2) with *o*-aminobenzoic acid the maximum intensity of colour is not reached until 2 h after coupling, and 15 min after coupling only about 20% of the maximum intensity has been attained. When a mixture of *o*- and *p*-aminobenzoic acids is examined, readings are made 15 and 120 min after coupling and by reference to suitable standards, the quantities of each may then be calculated.

Prior to diazotisation and coupling it is necessary to effect a separation of the aminobenzoic acids from urine and also to eliminate interfering substances. BROWN AND PRICE[11] have used ion-exchange resins for this purpose. In the present investigation, the acid urine is extracted with ether and the extracts, which contain interfering substances, are rejected. The residual urine is adjusted to pH 3.0 and again extracted with ether. The residue from these ether extracts is then examined by the diazotisation and coupling technique—the result being indicative of the aminobenzoic acid content.

Method. 10 ml of acid-hydrolysed urine are introduced into a 50-ml glass-stoppered measuring cylinder. Four successive extractions with 40 ml quantities of ether are made. After the addition of ether, the contents of the cylinder are shaken vigorously for 2 min; the ether extract is then removed with a teat pipette and discarded. Then 0.5 g of glycine is added to the urine residue and the pH adjusted to 3.0 by the addition of *N* sodium hydroxide. Extraction is then made with 80, 40 and 40 ml quantities of ether in a 100-ml glass-stoppered measuring cylinder. After the addition of ether, the contents are shaken vigorously for 2 min. The ether extracts are removed with a teat pipette, combined and evaporated to dryness.

To the residue are added 10 ml of water, 0.2 ml of 10 *N* hydrochloric acid and 1 ml of 0.1% sodium nitrite. After standing for 5 min, 1 ml of 0.5% ammonium sulphamate is added. After standing for 5 min, 1 ml of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution is added. Readings are made against a blank at 540 mμ, 15 and 120 min after the addition of the coupling reagent. Calculation of the *o*- and *p*-aminobenzoic acid contents is made by reference to appropriate standards.

Standards containing 10, 20 and 40 μg of *p*-aminobenzoic acid and 20, 40 and 80 μg of *o*-aminobenzoic acid are set up at the same time, the complete procedure being carried out.

References p. 419

o-Aminobenzoic acid, 20 to 80 μg, added to 10 ml of urine could be recovered with an accuracy of 79 to 89%. *p*-Aminobenzoic acid, 10 to 40 μg, added to 10 ml of urine could be recovered with an accuracy of 81 to 91%.

o-Hydroxyamines

Determinations are carried out colorimetrically with 2,6-dichloroquinone chlorimide. Alternatively one can employ the yellow colour developed by substances with an *o*-hydroxyamic grouping in the presence of nitrous acid but the reaction is not as sensitive.

Extraction from urine and the separation as far as possible from interfering substances *e.g.* phenols, phenolic acids, is an essential preliminary step. Initially the acidified urine (*N* with respect to hydrochloric acid) is extracted with ether, interfering phenols and phenolic acids being thereby removed. From the residual urine, 3-hydroxyanthranilic may be extracted with ether, the pH having been adjusted to 3.0. From the remaining urine after this procedure, *o*-aminophenol may be extracted with ether, the pH having been adjusted to 7.0.

Both 3-hydroxyanthranilic acid and *o*-aminophenol react with 2,6-dichloroquinone chlorimide to produce a compound which may be extracted into *n*-butanol to produce a blue-green colour. The maximum colour is developed within 5 min. The reaction is allowed to proceed at a pH below 8, destruction of the reagent being thereby reduced to a minimum. The reaction involving 3-hydroxyanthranilic acid is carried out at pH 7.6. This pH is not critical, similar results being obtained over the range pH 7 to 8. The reaction involving *o*-aminophenol is carried out at pH 7.9. This pH is critical. When the pH is much below this figure, the blue-green colour is not stable and changes rapidly to a yellow. Evaluations can be carried out in terms of the yellow colour but the writer considers that this lacks specificity. 3-Hydroxykynurenine, which is not extractable by ether, also exhibits this phenomenon.

The following are the details of the procedure:

Reagents. (1) 0.4% 2,6-dichloroquinone chlorimide in ethanol; (2) sodium bicarbonate solution, pH 7.9. Before use, 0.1 *N* hydrochloric acid is added to a saturated solution of sodium bicarbonate, the pH being checked with the glass electrode; (3) borate buffer solution, pH 7.6, checked by the glass electrode. Boric acid, 3.1 g; potassium chloride, 6.2 g; sodium chloride, 100 g; *N* sodium hydroxide, 4 ml; water, to 1000 ml; (4) *n*-butanol, AnalaR; (5) saturated sodium acetate.

3-Hydroxyanthranilic acid

10 ml of acid-hydrolysed urine are extracted four times with 40 ml quantities of ether. Extractions are carried out in a 50-ml glass-stoppered measuring cylinder. The contents are shaken vigorously for 2 min and after separation, the ether extract is removed with a teat pipette and discarded.

To the residual urine, 1 ml of saturated sodium acetate solution is added and the pH adjusted to 3.0 by the addition of *N* sodium hydroxide. The mixture is extracted with 40, 20 and 20 ml quantities of ether. Extractions are carried out as described above. The combined ether extracts are evaporated to dryness in an all-glass still.

The residue is dissolved in 5 ml of borate buffer solution (pH 7.6) and 0.5 ml of chlorimide reagent added. After standing at room temperature for 5 min, 5 ml of *n*-butanol are added and the mixture shaken vigorously for 2 min. After separation

References p. 419

and with the minimum of delay the butanol extract is separated and read against *n*-butanol at 600 mμ. A blank is carried out at the same time.

Standards containing 20, 50 and 100 μg 3-hydroxyanthranilic acid should be set up at the same time, the complete procedure being carried out.

o-Aminophenol

A preliminary extraction of the acid urine is carried out as described above.

To the residual urine, solid sodium bicarbonate is added until pH 7.0 is attained. The mixture is then extracted with 40, 20 and 20 ml quantities of ether, the extractions being carried out as described above. 6 drops of 10 *N* hydrochloric acid are added to the combined ether extracts which are then evaporated to dryness in an all-glass still.

The residue is dissolved in 5 ml of sodium bicarbonate solution (adjusted to pH 7.9) and 0.5 ml of chlorimide reagent added. After standing at room temperature for 5 min, the mixture is shaken vigorously for 2 min with 5 ml of *n*-butanol. The *n*-butanol extract is separated without delay and read against *n*-butanol at 600 mμ. A blank is carried out at the same time.

Standards containing 10, 20 and 50 μg of *o*-aminophenol are examined at the same time, the complete procedure being carried out.

3-Hydroxyanthranilic acid (10, 20, 50 and 100 μg) added to 10 ml of urine could be recovered with an accuracy of 79 to 91%.

o-Aminophenol (10, 20, and 50 μg) added to 10 ml of urine could be recovered with an accuracy of 82 to 91%.

p-Aminophenol

Some determinations of *p*-aminophenol have been made on acid-hydrolysed urine. Ether extracts were prepared exactly as described for the determination of *o*-aminophenol. Colorimetric estimations were then carried out as described previously[19].

RESULTS AND DISCUSSION

3-Hydroxyanthranilic acid, kynurenine, *o*-aminobenzoic acid and *o*-aminophenol have been determined in a number of urines. The results are recorded in Table I. The values recorded for the excretion of 3-hydroxyanthranilic acid, kynurenine and *o*-aminobenzoic acid by the normal subjects are considerably lower than those recorded by BOYLAND AND WILLIAMS[7] but are similar to those reported by BROWN AND PRICE[11], who used methods different from those described in this paper. It is of interest that the urinary excretion of tryptophan as determined by microbiological methods is considerably greater than that of any of the known metabolites[20–22].

The results recorded for the excretion of these tryptophan metabolites in a few cases of malignant disease of the bladder indicate that increased excretion may occur in some cases but is not general. The results are considerably lower than those recorded by BOYLAND AND WILLIAMS[7]. It is of interest that an increase of excretion of only certain metabolites can occur. An increased excretion of *o*-aminophenol occurred in some cases.

The results obtained in a single case of hypoplastic anaemia are of some interest. An increase in the excretion of 3-hydroxyanthranilic acid and kynurenine was noted

References p. 419

TABLE I

TRYPTOPHAN METABOLITES IN URINE

The results are expressed in mg/day

	3-Hydroxy-anthranilic acid	*o-Amino-phenol*	*Kynurenine*	*o-Amino-benzoic acid*
Normal				
1	8.3	8.9	3.3	1.1
2	9.4	3.9	1.8	1.9
3	12.5	6.3	4.9	2.6
4	12.5	10.0	3.6	1.6
5	12.0	6.4	3.1	1.3
6	7.6	7.3	2.1	2.4
7	9.1	8.4	1.9	1.8
8	6.3	8.2	3.2	2.3
	(*p*-aminobenzoic acid		1.3 to 2.1 mg/day)	
Hypoplastic anaemia				
	20.9	9.2	31.6	2.7
Carc. bladder				
1	5.7	7.2	1.8	1.3
2	32.0	29.4	2.6	1.8
3	32.0	16.0	8.4	2.3
4	28.4	23.6	1.2	1.6
5	14.3	22.5	1.3	1.6
6	23.0	11.5	2.3	1.8
7	14.2	9.5	2.8	1.6

but a normal excretion of *o*-aminobenzoic acid was obtained. This suggests that with regard to tryptophan metabolism, a constant pattern need not be expected in this condition.

The increased urinary excretion of tryptophan metabolites in malignant disease of the bladder and in hypoplastic anaemia may be explained as due to increased protein katabolism, either local or general. It could be a secondary rather than a primary factor in the causation of the disease, and the pattern of tryptophan metabolites excreted, could depend on conditions not necessarily connected with the primary cause of the disease.

The following evidence should support the assumption that 3-hydroxyanthranilic acid, *o*-aminophenol, *o*- and *p*-aminobenzoic acids and kynurenine do occur in human urine and were being determined by the appropriate methods.

3-Hydroxyanthranilic acid

1. it is extracted from aqueous solution by ether at pH 3,

2. it reacts with 2,6-dichloroquinone chlorimide to produce a blue green colour soluble in *n*-butanol,

3. when appropriate urine extracts are examined by paper chromatography (*n*-butanol–acetic acid–water),

References p. 419

(a) the R_F is similar to that of authentic 3-hydroxyanthranilic acid,
(b) a yellow colour is produced in the presence of nitrous acid,
(c) a brown colour is produced in the presence of ammoniacal silver nitrate,
(d) a blue fluorescence is produced in ultra-violet light.

o-Aminophenol

1. it is extracted from aqueous solution by ether at pH 7,
2. it reacts with 2,6-dichloroquinone chlorimide and the colour so produced is influenced by pH in the same manner as *o*-aminophenol,
3. when appropriate urine extracts are examined by paper chromatography (*n*-butanol–acetic acid–water),
(a) the R_F is similar to that of authentic *o*-aminophenol,
(b) a yellow colour is produced in the presence of nitrous acid,
(c) a brown colour is produced in the presence of ammoniacal silver nitrate,
(d) does not fluoresce in ultra-violet light.

Aminobenzoic acids

When appropriate urine extracts are examined by paper chromatography (*n*-butanol–acetic acid–water), R_F's are similar to those of authentic specimens of *o*- and *p*-aminobenzoic acids and produce the appropriate diazotisation and coupling reactions.

Kynurenine

The production by the action of hot alkali of a volatile aromatic amine with a substituent group in the *ortho* position and the properties of a ketone.

In Table II A are recorded the result of the oral ingestion of 5 g of *l*-tryptophan by the normal upon the urinary excretion of 3-hydroxyanthranilic acid, kynurenine, *o*-aminobenzoic acid and *o*-aminophenol. There are significant increases in the excretion of 3-hydroxyanthranilic acid, kynurenine and *o*-aminobenzoic acid but a very large amount of tryptophan remains unaccounted for. BROWN AND PRICE[11] reached similar conclusions. There appeared to be no effect upon the excretion of *o*-aminophenol which would suggest that this substance is not a metabolite of tryptophan.

In Table II B are recorded the results of the oral ingestion of 200 mg of acetanilide by the normal upon the urinary excretion of *o*- and *p*-aminophenol. Only the excretion of *p*-aminophenol was increased. This is in agreement with the findings of BRODIE AND AXELROD[23] who suggest that in the human, aniline and related compounds are hydroxylated in the *para* rather than the *ortho* position.

The origin of the *o*-aminophenol present in urine is obscure. It could have been produced as the result of a decarboxylation by tissue or bacterial enzymes. The latter could occur either in the alimentary tract or in an infected urine. The substrate for such a decarboxylation could be 3-hydroxyanthranilic acid or 3-hydroxy-4-aminobenzoic acid, both *o*-hydroxyamines. The latter substance could result from the hydroxylation of *p*-aminobenzoic acid, the formation of a *p*-hydroxyamine cannot take place since the position *para* to the amino group is already occupied (*cf.* aniline). Since some known carcinogens possess an *o*-hydroxyamine structure, it has been suggested that this property may be shared by such simple compounds as *o*-aminophenol[6].

References p. 419

TABLE II

A. THE URINARY EXCRETION OF 3-HYDROXYANTHRANILIC ACID, KYNURENINE, *o*-AMINOBENZOIC ACID, AND *o*-AMINOPHENOL BY THE NORMAL AFTER THE ORAL INGESTION OF 5g OF *l*-TRYPTOPHAN

The results are expressed in mg/day

	3-Hydroxy-anthranilic acid	*o-Amino-phenol*	*Kynurenine*	*o-Amino-benzoic acid*
1. A	8.1	7.6	2.3	1.1
B	18.0	8.5	4.5	2.5
2. A	7.8	6.8	1.9	0.9
B	16.0	7.4	5.1	3.1
3. A	9.2	7.4	1.9	1.3
B	20.6	7.9	6.2	3.0

A – control
B – after tryptophan

B. THE URINARY EXCRETION OF *o*- AND *p*-AMINOPHENOL BY THE NORMAL AFTER THE INGESTION OF 200 mg ACETANILIDE

The results are expressed in mg/day

	o-Aminophenol	*p-Aminophenol*
1. A	8.2	1.3
B	9.8	56.0
2. A	6.4	1.1
B	7.8	64.6
3. A	8.2	0.9
B	9.0	49.4

A – control
B – after acetanilide

It is quite apparent that a considerable amount of investigation, both chemical and biological, is essential before our knowledge concerning the metabolism of tryptophan is anything like complete and its role, if any, in malignancy established.

Normal urine appears to contain a small amount of *p*-aminophenol (about 1 mg/day) but its origin is obscure. It and the unknown volatile diazotisable amines (obtained by the distillation of neutral urine) could possibly be derived from the metabolism of artificial colouring materials added to foodstuffs—those of the Ponceau series could thus be implicated. The former could be derived from those prepared from diazotised aniline and the latter from diazotised products of more complex aromatic amines.

SUMMARY

1. Methods have been described for the determination of 3-hydroxyanthranilic acid, kynurenine, *o*-aminobenzoic acid and *o*-aminophenol in urine.

2. The urinary excretion of these substances has been studied in the normal, one case of hypoplastic anaemia and a few cases of malignant disease of the bladder.

References p. 419

3. The effect of the ingestion of 5 g of *l*-tryptophan upon the excretion of these substances in the normal has been studied.

4. The effect of the ingestion of acetanilide upon the urinary excretion of *o*- and *p*-aminophenol has been studied in the normal.

REFERENCES

1 L. MUSAJO, C. A. BENASSI AND A. PARPAJOLA, *Clin. Chim. Acta*, 1 (1956) 229.
2 C. E. DALGLEISH AND S. TEKMAN, *Biochem. J.*, 56 (1954) 458.
3 C. E. DALGLEISH, *Biochem. J.*, 61 (1955) 334.
4 C. E. DALGLEISH, *Biochem. J.*, 61 (1955) 328.
5 G. M. BONSER, D. B. CLAYSON, J. W. JULL AND L. N. PYRAH, *Brit. J. Urol.*, 26 (1954) 49.
6 A. L. WALPOLE, M. H. C. WILLIAMS AND D. C. ROBERTS, *Brit. J. Ind. Med.*, 9 (1952) 255.
7 E. BOYLAND AND D. C. WILLIAMS, *Biochem. J.*, 64 (1956) 578.
8 E. BOYLAND AND G. WATSON, *Nature*, 177 (1956) 837.
9 K. J. ALTMAN AND G. MILLAR, *Nature*, 172 (1953) 868.
10 E. BOYLAND, D. M. WALLACE AND D. C. WILLIAMS, *Brit. J. Urol.*, 27 (1955) 11.
11 R. R. BROWN AND J. M. PRICE, *J. Biol. Chem.*, 219 (1956) 985.
12 D. COPPINI, *Mikrochim. Acta*, 12 (1956) 1818.
13 A. C. BRATTON AND E. K. MARSHALL JR., *J. Biol. Chem.*, 128 (1939) 537.
14 S. O. OTANI, N. NISHINO AND K. IMAI, *Z. physiol. Chem.*, 270 (1941) 60.
15 Y. KOTAKE AND M. KAWASE, *Z. physiol. Chem.*, 214 (1933) 6.
16 C. C. PORTER, I. CLARK AND R. H. SILBER, *J. Biol. Chem.*, 167 (1947) 573.
17 M.ŠPAČEK, *Canad. J. Biochem. and Physiol.*, 32 (1954) 604.
18 R. LEMBERG, D. TANDY AND N. E. GOLDSWORTHY, *Nature*, 157 (1946) 103.
19 S. L. TOMPSETT, *Clin. Chim. Acta*, 3 (1958) 149.
20 H. W. WOODSON, S. W. HIER, J. D. SOLOMON AND O. BERGEIM, *J. Biol. Chem.*, 172 (1945) 613.
21 R. D. ECKHARDT AND C. S. DAVIDSON, *J. Biol. Chem.*, 177 (1949) 687.
22 S. L. TOMPSETT AND J. FITZPATRICK, *Brit. J. Exptl. Pathol.*, 31 (1950) 70.
23 B. B. BRODIE AND J. AXELROD, *J. Pharmacol. Exptl. Therap.*, 94 (1948) 22.

Received October 20th, 1958

PHYSICO-CHEMICAL STUDIES ON FOUR MACROGLOBULINS

A. E. STEEL

Biochemistry Department, Killingbeck Hospital, Leeds (Great Britain)

The formation of a precipitate of protein when certain sera are diluted with water was described by BRAHMACHARI AND SEN[1] and by SIA[2]. WALDENSTRÖM[3] diluted serum 1 in 16 and observed the bulk and appearance of the precipitate. Many other workers have used the "water–euglobulin test" as a screening test for macroglobulinaemia. MACKAY *et al.*[4] state that false positives are obtained notably in multiple myeloma while BICHEL, BING AND HARBOE[5], LILJESTRAND AND OLHAGEN[6], SANDKÜHLER[7], FERRIMAN AND ANDERSON[8] and MACKAY *et al.*[9] report negative results with sera containing macroglobulins. The stability of some proteins has been reported. STEIN AND WERTHEIMER[10] state that their "dilution fraction" is remarkably labile while LERNER AND GREENBERG[11] reported a cryoglobulin as being stable over a long period. Electrophoresis of macroglobulins has been recorded. Many reports show migration with the γ-globulins while MACKAY *et al.*[4], FERRIMAN AND ANDERSON[8] and BICHEL, BING AND HARBOE[5] report β-globulin mobility and SEHON *et al.*[12] record β- and α_2-mobility. JIM AND STEINKAMP[13] and SEHON[12] report macroglobulins showing a positive periodic acid–Schiff staining reaction for protein-bound polysaccharide. Ultraviolet absorption spectra have been examined by LERNER AND GREENBERG[11], LERNER, BARNUM AND WATSON[14], BARR, READER AND WHEELER[15] and MANDEMA, VAN DER SCHAAF AND HUISMAN[16] giving data for cryoglobulins and macroglobulins indicating a similarity to the γ-globulins. GRÜMER, GRUNZE AND HÖRNER[17] compared the spectra of three macroglobulins with γ-globulin and indicated a reduced tryptophan and tyrosine content. The isoelectric precipitation of euglobulins was reported by GREEN[18] and by SANDOR AND CHEDDAHA[19]. SANDOR[20] and VARGUES AND LABROSSE[21] describe a euglobulin having an isoelectric point between pH 7.0 and 7.6 while JIM AND STEINKAMP[13] record the maximum insolubility of a macroglobulin at pH 6.4. The present paper describes some properties of four macroglobulins.

MATERIALS AND METHODS

Sera from a number of patients with rheumatic diseases have been encountered which on dilution with water yield a precipitate of protein (euglobulin). Case 1 (AK) was first observed in November 1953 when the water precipitable euglobulin accounted for 1.75 g per 100 ml of serum. Eight months later a concentration of 2.80 g/100 ml was recorded and in August 1957 the concentration was 3.90 g/100 ml. The serum albumin had ranged from 1.1 to 1.4 g/100 ml and the total γ-globulins from 2.4 to 6.5 g/100 ml of serum during this period. Over a period of three years a survey of a large number of sera from patients attending a Rheumatic Clinic revealed nine further cases giving a positive "water" test in which serum euglobulin assays ranged from 0.41 to 1.48 g/100 ml. Three of these cases showing increased serum γ-globulins (case 2 HC 2.1 g/100 ml; case 3 NG 3.4 g/100 ml; case 4 AMP 2.0 g/100 ml) together with case 1

References p. 423

(AK) were examined in the ultracentrifuge. The sera were found to contain macroglobulins. The euglobulins from these four cases were examined by a variety of techniques.

EXPERIMENTAL

Precipitation of euglobulin from the sera examined reached a maximum after 15 min in the dilution range 1 in 20 to 1 in 50 where a nearly linear relation was obtained between dilution and optical density at 590 mμ. Flocculation usually occurred within 30 min. Quantitative assays were carried out by adding 0.2 ml of serum to 4.8 ml of water and measuring the optical density after 15 min. 0.05 ml of a saturated solution of sodium chloride was added to dissolve the globulin and the resulting optical density subtracted from the first reading. Assayed protein solutions precipitated with sulphosalicylic acid were used as standards. Assays performed by centrifuging off the precipitate from 0.5 ml of serum after dilution with 15 ml of water and determination of the protein by a micro-Kjeldahl method showed close agreement with the turbidimetric analysis.

Stability of macroglobulin

Judged by the amount of precipitate produced on dilution with water, sera remain stable when stored for at least one year at —25°. They are stable at room temperature for several days but are unstable at 37°. Sera repeatedly thawed at 37° from the frozen state showed a progressive reduction in the amount of "euglobulin" precipitated. Freeze dried sera stored at room temperature for up to one year and initially containing 1.20 and 0.86 g euglobulin/100 ml gave no precipitation after reconstitution and dilution with water.

Electrophoresis

Isolated macroglobulin was dissolved in veronal buffer (pH 8.6, 0.1 *M*) and examined by paper electrophoresis. Papers were dried at 110° and stained with bromophenol blue, sudan black and with periodic acid–Schiff stain using the technique of KÖIW AND GRÖNWALL [22]. Results are given in Table I. Starch gel electrophoresis was

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF THE SERUM AND ISOLATED MACROGLOBULINS FROM THE FOUR CASES

Case ref.	*Sex*	*Age years*	*Whole serum*		*Isolated protein*				
					Electrophoresis			*Ultra-violet*	
			Viscosity millipoise at 20°	*Macroglobulin g/100 ml*	*Mobility*	*PAS*	*Sudan black*	λ_{max} *mμ*	*E 1% 1 cm at* λ_{max}
1 (AK)	F	29	45.8	2.80	γ-globulin	+	—	278	14.3
2 (HC)	F	55	30.2	1.22	γ-globulin	+	—	279	13.4
3 (NG)	F	55	29.3	1.48	γ-globulin	+	—	278	13.5
4 (AMP)	F	58	28.5	0.48	γ-globulin	+	—	278	14.0

carried out using hydrolysed potato starch at a concentration of 22% w/v in 0.03 *M* borate buffer at pH 8.6. Intense staining at the point of insertion of the sample serum or protein was seen consistent with the observations of SILBERMAN [23]. The patterns for whole serum showed no otherwise abnormal characteristics.

References p. 423

Ultra-violet absorption

Clear solutions of the macroglobulins were prepared in 1% v/v hydrochloric acid solution at a final protein concentration of 50 mg/100 ml and optical densities measured in the range 240 to 300 mμ using silica cells in a "Unicam" S.P. 500 spectrophotometer. Curves for the four macroglobulins and for γ-globulin are shown in Fig. 1.

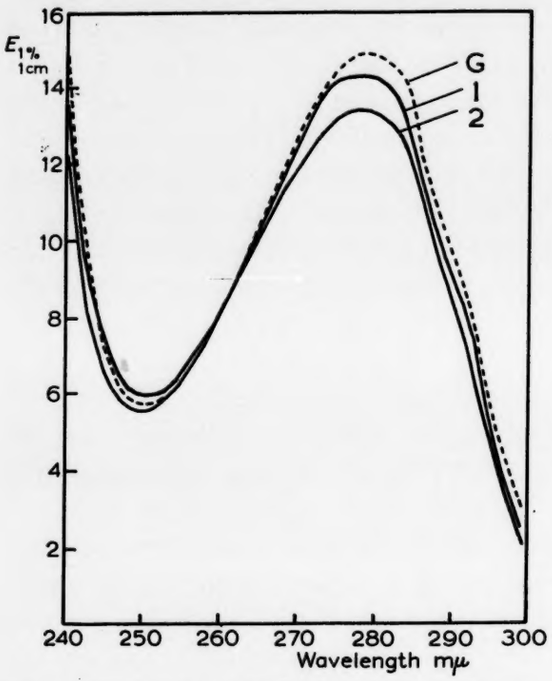


Fig. 1. Ultra-violet absorption curves. Curve G: Human γ-globulin, Curve 1: Macroglobulins from cases AK and AMP, Curve 2: Macroglobulins from cases NG and HC.

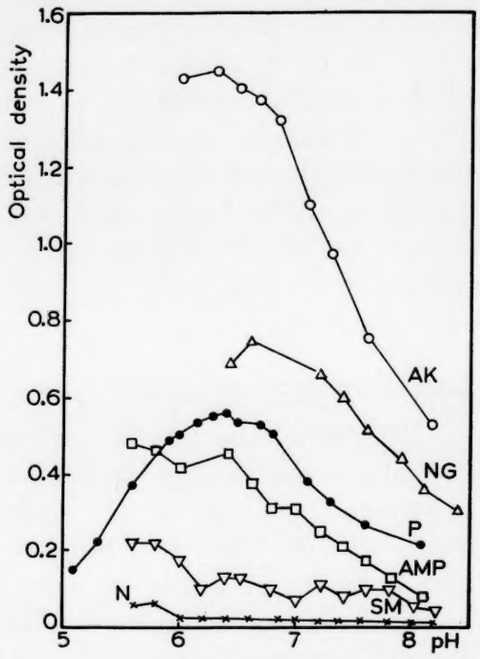


Fig. 2. Optical density produced by mixing serum (1 vol.) from cases of macroglobulinaemia with buffer (40 vols., 0.02 *M*) over a range of pH values (curves AK, NG, and AMP). Curve P is given by a saline solution of the protein isolated from case AK, curve N with a normal serum and curve SM from a serum containing a 7S-euglobulin.

pH precipitation curves

Measured volumes of serum were added to buffer solutions ($M = 0.02$) in the pH range of 5 to 9 to give a final dilution of 1 in 40. The optical density of the mixtures was measured after 15 min at 590 mμ. Fig. 2 illustrates the results obtained with sera containing macroglobulin, an isolated macroglobulin, a 7S-euglobulin and normal human serum.

DISCUSSION

A positive water dilution test combined with a very high serum viscosity indicates the possible presence of high molecular weight proteins. The four sera examined in this paper had these properties and contained macroglobulins when examined in the ultracentrifuge (see STEEL[24], for data). The macroglobulins appear to be stable when stored at low temperatures but are unstable at 37°. It was frequently observed that solutions of macroglobulins yield precipitates of protein which are difficult to redissolve on storing at 2° or at room temperature. One stored sample after redissolving gave only a single slow peak in the ultracentrifuge. Some samples of macroglobulin

References p. 423

were found to dissolve on heating, an observation also noted by SANDKÜHLER[7]. Analysis by electrophoresis at pH 8.6 showed these globulins to possess low mobility. Tests at other pH values (pH 4.5 to 10) indicated slower migration that other serum proteins. A stippled effect at the origin on the paper strips was noted in some experiments and may be due to precipitation of the macroglobulin at this point. All four macroglobulins showed a strong PAS staining reaction for glycoprotein. From the apparent failure to penetrate starch gel blocks on electrophoresis it would appear that these γ-macroglobulins combine size and asymmetry in their molecules since the normal S-19 α_2-glycoprotein of serum migrates into the gel. It will be demonstrated in a subsequent report that these macroglobulins show very high viscosities in solution. All the sera gave marked precipitation in the region of pH 6.3 to 6.5, a saline solution of one of the globulins gave a peak precipitation at pH 6.4. Ultra-violet absorption analysis indicated a similarity to the γ-globulins although extinctions were slightly lower.

SUMMARY

Euglobulins containing macroglobulins have been isolated from four sera by dilution with water and examined for stability, electrophoretic mobility and staining reaction, ultra-violet absorption and isoelectric precipitation.

REFERENCES

[1] U. N. BRAHMACHARI AND P. B. SEN, *Indian Med. Gaz.*, 58 (1923) 295.
[2] R. H. P. SIA, *China Med. J.*, 38 (1924) 35.
[3] J. WALDENSTRÖM, *Acta Med. Scand.*, 117 (1944) 216.
[4] I. R. MACKAY, L. I. TAFT AND E. F. WOODS, *Brit. Med. J.*, 1 (1957) 561.
[5] J. BICHEL, J. BING AND N. HARBOE, *Acta Med. Scand.*, 138 (1950) 1.
[6] A. LILJESTRAND AND B. OLHAGEN, *Acta Med. Scand.*, 151 (1955) 425.
[7] St. SANDKÜHLER, *Klin. Wochschr.*, 33 (1955) 536.
[8] D. G. FERRIMAN AND A. B. ANDERSON, *Brit. Med. J.*, 2 (1956) 402.
[9] I. R. MACKAY, N. ERIKSEN, A. G. MOTULSKY AND W. VOLWILER, *Am. J. Med.*, 20 (1956) 564.
[10] L. STEIN AND E. WERTHEIMER, *Ann. Trop. Med. Parasitol.*, 36 (1942) 17.
[11] A. B. LERNER AND G. R. GREENBERG, *J. Biol. Chem.*, 162 (1946) 429.
[12] A. H. SEHON, L. GYENES, J. GORDON, M. RICHTER AND B. ROSE, *J. Clin. Invest.*, 36 (1957) 456.
[13] R. T. S. JIM AND R. C. STEINKAMP, *J. Lab. Clin. Med.*, 47 (1956) 540.
[14] A. B. LERNER, C. P. BARNUM AND C. J. WATSON *Am. J. Med. Sci.*, 214 (1947) 416.
[15] D. P. BARR, G. G. READER AND C. H. WHEELER, *Ann. Internal Med.*, 32 (1950) 6.
[16] E. MANDEMA, P. C. VAN DER SCHAAF AND T. H. J. HUISMAN, *J. Lab. Clin. Med.*, 45 (1955) 261.
[17] H. D. GRÜMER, E. GRUNZE AND H. HÖRNER, *Klin. Wochschr.*, 33 (1955) 588.
[18] A. A. GREEN, *J. Am. Chem. Soc.*, 60 (1938) 1108.
[19] G. SANDOR AND L. CHEDDAHA, *Bull. soc. chim. biol.*, 31 (1949) 1525.
[20] G. SANDOR, *Bull. soc. chim. biol.*, 33 (1951) 1483.
[21] R. VARGUES AND S. LABROSSE, *Presse méd.*, 62 (1954) 1740.
[22] E. KÖIW AND A. GRÖNWALL, *Scand. J. Clin. & Lab. Invest.*, 4 (1952) 244.
[23] H. J. SILBERMAN, *Lancet*, 2 (1957) 26.
[24] A. E. STEEL, *Clin. Chim. Acta*, (1959) in the press.

Received November 7th, 1958

INVESTIGATIONS ON THE INFLUENCE OF TOLBUTAMIDE ON CARBOHYDRATE METABOLISM. III

B. LEIJNSE, H. J. YBEMA

Biochemical Laboratory, Coolsingel Hospital, Rotterdam

AND

CHR. E. KAMMINGA

Clinical-Chemical Laboratory, Calandstraat, Rotterdam (The Netherlands)

Experiments on the influence of the oral antidiabetic agent tolbutamide on various aspects of the carbohydrate metabolism of rats have been described by two of us[1, 2]. Some of the effects observed were not consistent with the theory that administration of tolbutamide results in a stimulation of insulin production and/or secretion by pancreatic islet tissue.

In a recent communication VON HOLT *et al.*[3] reported that a single dose of the related substance carbutamide (300 mg/kg body weight) increased the insulin content of rats' serum from 29 to 170 mU/ml on the average. They performed the insulin assay using isolated rat diaphragms according to the method described by RANDLE[4]. Evidently other substances besides insulin are able to stimulate the glucose utilization by the diaphragm.

Without giving experimental details, VON HOLT[5] states that carbutamide is inactive in this respect. On the other hand, MOHNIKE *et al.*[6] have observed, in the case of added tolbutamide, a stimulating effect on the glucose utilization and oxygen consumption and an inhibitory effect of added carbutamide or tolbutamide on the glycogen synthesis by diaphragm tissue.

In this paper experiments will be described in which were studied:

(a) the influence of tolbutamide on carbohydrate utilization by the isolated rat diaphragm, and

(b) the influence of oral administration of tolbutamide on the insulin content of rat blood serum.

METHODS

The technique used was essentially that of GROEN *et al.*[8]. Tolbutamide was dissolved as its sodium salt in modified GEY AND GEY[9] buffer. For the other techniques used we refer to our previous publications[1, 2].

EXPERIMENTAL RESULTS

The results of our experiments on the utilization of glucose, fructose and galactose by isolated rat diaphragm are shown in Table I. It could be demonstrated that the addition of tolbutamide to the incubation fluid stimulated glucose and fructose utilization.

References p. 426

TABLE I

THE INFLUENCE OF TOLBUTAMIDE ON THE UTILIZATION OF GLUCOSE, FRUCTOSE AND GALACTOSE BY RAT DIAPHRAGMS IN mg PER g TISSUE

The volume of incubation fluid was 1 ml, the hexose concentration 150 mg%, the tolbutamide concentration 25 mg%. One half diaphragm was used in each experiment, the other hemidiaphragm of the same animal was the control. Incubation time 90 min, temperature 37°.

Hexose used	*Number of experiments*	*Average utilization*		*Increase*	
		control series mg/g	*tolbut. series mg/g*	*mg/g*	*p-value*
Glucose	54	4.81	5.27	0.46 ± 0.22	0.04
Fructose	16	2.36	2.89	0.53 ± 0.21	0.03
Galactose	6	0.12	0.15	0.03 ± 0.31	0.90

The results of estimations of insulin activity in blood serum of rats, which had or had not previously received oral doses of tolbutamide, are summarized in Table II*.

TABLE II

THE INFLUENCE OF TOLBUTAMIDE ADMINISTRATION ON THE INSULIN CONTENT OF RAT SERUM

The rats received 50 mg of tolbutamide and were sacrificed 2½ h later. The serum of each rat was collected separately, together with a control series of an equal number of untreated rats. In the experiment the serum was diluted 5 times with the buffer used. Experimental conditions as in Table I.

Expt.	*Addition*	*Number of experiments*	*Average utilization mg/g*	*Increase*	
				mg/g	*p-value*
A	none 10^{-4}U Ins./ml	10	6.04 6.96	0.92 ± 0.32	0.02
B	none control serum	6	6.50 7.55	1.05 ± 0.18	0.005
C	control serum experimental serum	24	6.94 6.50	—0.44 ± 0.38	0.30

A. An insulin concentration of 10^{-4}U/ml incubation mixture could be demonstrated.

B. The presence of rat serum in the incubation fluid produced a definite insulin-like effect on the glucose utilization of the diaphragms. From the magnitude of the effect, the 'insulin' content of the serum can be estimated to be about 10^{-3}/ml.

C. Oral administration of tolbutamide to the rats did not increase the insulin activity of their serum.

DISCUSSION

From our results it can be seen that on studying the effect of tolbutamide by means of the glucose utilization by incubated rat diaphragm tissue, we could not confirm the results of VON HOLT[5]. In accordance with the results obtained by MOHNIKE with tolbutamide[6] and by LUNDBAEK *et al.* with carbutamide and tolbutamide[7], we found that the glucose utilization of diaphragm tissue is stimulated by the addition of

* We are indebted to Dr. J. LENS of Organon Laboratories Inc. for a gift of pure insulin and a number of animals.

References p. 426

the antidiabetic substance directly to the incubation medium. In the case of fructose utilization, the LUNDBAEK group found that carbutamide had a similar effect, while our results indicated the same for tolbutamide.

The stimulation of glucose and fructose utilization by diaphragm tissue by the addition of tolbutamide to the incubation medium is fairly small and, as appeared from the p-values, rather variable.

As far as galactose utilization is concerned, LUNDBAEK again reported a stimulation by carbutamide, whereas we did not see any effect. However, in the case of this sugar a stimulation is difficult to establish, as the galactose uptake is small and a relatively large variation in the results of the diaphragm technique is inevitable.

The administration of tolbutamide to rats for the purpose of studying the effect on the insulin activity of their blood serum invariably resulted in a decrease of the blood glucose level of about 40%. Nevertheless we were unable to demonstrate the corresponding large increase in blood insulin activity as reported by VON HOLT *et al.* after carbutamide treatment.

We are aware of the fact that the interpretation of experiments with isolated diaphragms requires careful handling of the data obtained. There are a number of unexplained facts in this technique. Estimated by the method of GROEN *et al.*[8], the insulin content of normal human serum is found to be 0.1–3 mU/ml. Estimated by the method of RANDLE[4], the results are 10–20 mU/ml. The same lack of agreement is found between the results of the experiments of VON HOLT *et al.* and our results with normal rat serum.

However, it is impossible at the moment to accept the results obtained by VON HOLT *et al.* as a definite proof for the hypothesis that administration of tolbutamide results in a stimulation of insulin production and/or secretion by pancreatic islet tissue.

SUMMARY

Experiments are described in which the influence of tolbutamide on the carbohydrate metabolism of the isolated rat diaphragm is studied. The addition of tolbutamide to the incubation medium resulted in a slight stimulation of glucose and fructose utilization by the tissue. In another group of experiments the influence of the tolbutamide treatment of rats on the insulin content of their blood serum was studied with the diaphragm technique. No effect could be established, though the tolbutamide administration invariably resulted in a decrease of the blood sugar level of about 40%.

REFERENCES

[1] B. LEIJNSE AND H. J. YBEMA, *Clin. Chim. Acta*, 2 (1957) 284.
[2] B. LEIJNSE AND H. J. YBEMA, *Clin. Chim. Acta*, 3 (1958) 143.
[3] C. VON HOLT, L. VON HOLT, J. KRACHT, B. KRÖNER AND J. KÜHNAU, *Science*, 125 (1957) 735.
[4] P. J. RANDLE, *Brit. Med. J.*, (1954 I) 1237.
[5] C. VON HOLT, L. VON HOLT AND B. KRÖNER, *Naturwissenschaften*, 43 (1956) 162.
[6] G. MOHNIKE, K. W. KNITSCH, H. BOSER, G. WERNER AND S. WERNER, *Deut. med. Wochschr.*, 82 (1957) 1580.
[7] K. LUNDBAEK, K. NIELSEN AND O. J. RAFAELSEN, *Lancet*, 274 (1958) 1036.
[8] J. GROEN, C. E. KAMMINGA, A. F. WILLEBRANDS AND J. R. BLICKMAN, *J. Clin. Invest.*, 31 (1952) 97.
[9] G. O. GEY AND M. K. GEY, *Am. J. Cancer*, 27 (1936) 45.

Received December 30th, 1958

THE EFFECT OF SELF- AND EXTERNAL RADIATIONS ON ^{131}I-LABELLED L-THYROXINE AND 3,5,3′-TRIIODO-L-THYRONINE IN SOLUTION

JAMSHED R. TATA*

National Institute for Medical Research, Mill Hill, London (Great Britain)

The very low concentrations of thyroid hormones labelled with ^{131}iodine that have to be employed for studying their metabolic fate under physiological conditions have necessitated the preparation of L-thyroxine and 3,5,3′-triiodo-L-thyronine of extremely high specific radioactivity. Thus radioactive thyroxine and triiodothyronine of specific activities of 20–50 mC/μmole of each hormone are being commonly used for many investigations. That the presence of such large amounts of radio-iodine in almost pure solutions of the two iodothyronines could lead to their radiochemical decomposition was inferred from our observation that the amount of radioactive contaminants in such solutions was proportional to their specific activities and increased with time of storage. But for a few incomplete remarks on the stability of radioactive thyroid hormones[1, 2], no information has been published regarding the susceptibility of thyroxine and triiodothyronine to breakdown during the absorption of ionising radiation. Because the presence of radioactive contaminants in preparations of thyroid hormones could lead to subsequent erroneous interpretations in studies on their metabolic fate, it was felt necessary to undertake a systematic investigation of the production and nature of these artefacts. In the work reported here are described the stability of high-specific-activity labelled thyroxine and triiodothyronine as a function of time of storage, the identical radiochemical reactions obtained with self- and external radiation, the nature of degradation products and the prevention of their formation.

EXPERIMENTAL

For studies on the effect of self-radiation, ^{131}I-labelled L-thyroxine and 3,5,3′-triiodo-L-thyronine (labelled in the 3′,5′ and 3′ positions respectively) were obtained from Abbott Laboratories, Oak Ridge, Tennessee. The hormones were tagged by a method of radioisotopic exchange[3] resulting in a specific radioactivity of 20–30 mC/μmole for each compound. The usual concentration was 25–30 μg of iodothyronine/ml of 50% propylene glycol in unbuffered water.

Cylindrical vials, 1.5 cm in diameter, were used for storing 1.5–2.0 ml of each solution at $-2°$ to 0°. Under these conditions, the average β-radiation dose from 1 mC of labelled material was calculated to be $6.8 \cdot 10^3$ rads/day, $4 \cdot 10^4$ rads/8 days, $6 \cdot 10^4$ rads/16 days and $8 \cdot 10^4$ rads at infinity, on the basis of average ^{131}I β-radiation energy of 0.2 MeV and ignoring the γ-radiation (1 rad = 100 ergs absorbed/g of material). Since the radiation dose at the liquid surface is only half of the dose at the centre, the actual amount of radiation delivered was somewhat less because of an increasing surface : volume ratio with time, caused by withdrawal of small aliquots of

* Beit Memorial Fellow for medical research.

References p. 437

samples for analysis or other experiments during the storage period. To check the role played by the solvent in the radiochemical reaction, aliquots of high-specific-activity iodothyronines were stored for varying periods of time after removal of the 50% propylene glycol and redissolved immediately before analysis. Storage of thyroxine and triiodothyronine of feeble specific radioactivity (0.04–0.20 mC/μmole) under the same conditions as above also constituted control experiments. Stored samples were analyzed for radiochemical changes at 1–4 days' intervals up to 40 days.

The effects of external radiation were studied on ^{131}I-labelled thyroxine and triiodothyronine of very low specific radioactivity (0.01 mC/μmole and less) which were prepared by radio-iodination of 3,5-diiodo-L-thyronine. 0.5 ml and 1.5 ml of 50% propylene glycol solutions of 10^{-5} M–$2.5 \cdot 10^{-4} M$ thyroxine and triiodothyronine were irradiated for different lengths of time in tubes of 9 mm diameter. Two sources of external radiation were employed:

(1) High energy X-rays from a Metropolitan-Vickers 4 MeV Linear Accelerator at a dose rate of 1500 $\pm$ 10% rads/min.

(2) γ-rays from ^{60}cobalt (equal number of quanta of 1.17 and 1.33 MeV) at a dose rate of 164–170 rads/min. The solvent alone was also irradiated and radiothyroxine and radiotriiodothyronine were later added for storage.

The effect of radiation on the chemical composition of each sample was determined by quantitative paper chromatographic and electrophoretic analysis of the radio-iodinated constituents[4]. Chromatograms were developed on Whatman No. 1 paper using the following solvent systems in the ascending directions: (1) *n*-butanol–acetic acid–water (78:10:12); (2) *n*-butanol–dioxane (80:20) saturated with 2 N NH_4OH; (3) collidine–water (100:35.5) in atmosphere of NH_3. 0.05 M veronal buffer pH 8.6 and 0.075 M TRIS (Tris[hydroxymethyl]aminomethane) buffer pH 9.0 were used for electrophoretic analysis on Whatman No.3 paper. Thyroxine, triiodothyronine and compounds structurally related to them were added as "carriers" for analysis and their chromatographic and electrophoretic mobilities were also compared separately with radioactive products. Because of the small amounts of iodothyronines used in these experiments, it was not possible to study the nature of breakdown products by classical chemical methods. However, their identification was attempted by spot tests on paper by spraying specific organic group reagents on aliquots of the more abundant degradation products isolated by elution from paper after chromatographic separation. The composition of spot-test reagents and the method of their use have been described elsewhere[5, 6].

RESULTS

Effect of self-radiation

Storage of radioactive thyroid hormones of high specific activity led to a marked diminution in the fraction of iodothyronine radioactivity after a certain period of relative stability. The principal radio-iodinated compounds formed as a result of the breakdown of labelled thyroxine are seen in autoradiograms of chromatograms in Fig. 1.

Up to the 19th day of storage (the first day of storage was counted as 24 hours after the time of assay of radioactivity of labelled hormones by Abbott Laboratories) in the case of one particular sample of radiothyroxine illustrated in Fig. 1, about 90% of total radioactivity was still present as thyroxine with about 5–10% of it as

References p. 437

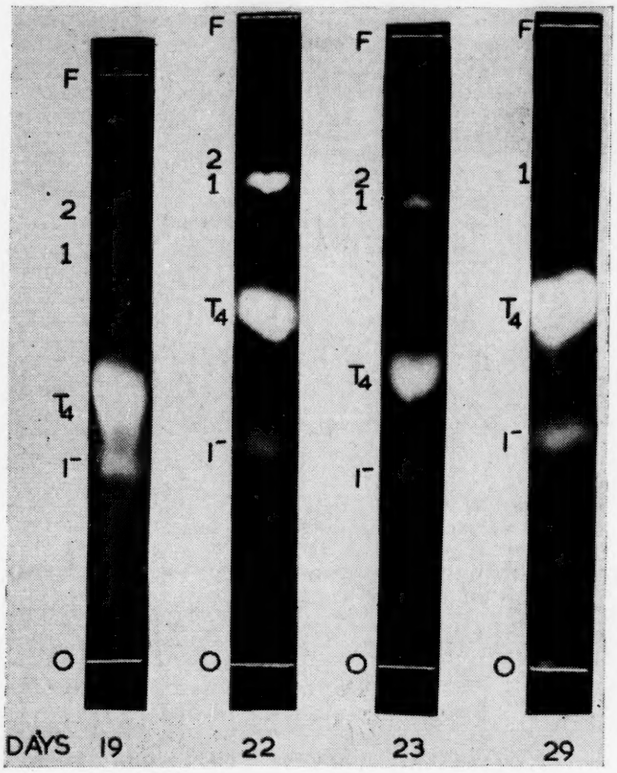


Fig. 1. Autoradiochromatograms of aliquots of the same sample of ^{131}I-labelled L-thyroxine analyzed on the 19th, 22nd, 23rd and 29th days of storage at $-1° \pm 2°$. Specific radioactivity: 23.0 mC/μmole of thyroxine. Chromatographic solvent: butanol–dioxane–ammonia. O = Origin of chromatogram; F = solvent front; T_4 = L-thyroxine; I^- = iodide; 1 and 2 = unknown radiochemical breakdown products.

Fig. 2. Radiochemical change brought about as a result of irradiation of ^{131}I-labelled (A) L-thyroxine and (B) 3,5,3′-triiodo-L-thyronine of low specific activity with X-rays from the 4 MeV Linear Accelerator. Specific radioactivity: 0.002 mC/μmole of thyroxine; 0.0016 mC/μmole of triiodothyronine. ^{131}I distribution before X-irradiation: (A) 91% in thyroxine, 9% in iodide; (B) 94% in triiodothyronine, 6% in iodide. Radiation dose: (A) 45,000 rads, (B) 60,000 rads. Chromatographic solvent: butanol–dioxane–ammonia. T_3 = 3,5,3′-triiodothyronine. Other abbreviations as in Fig. 1.

iodide. On the 22nd day of storage, however, a very significant amount of radioactivity appears in a new spot indicated as Compound "1" and which has a higher chromatographic mobility than that of thyroxine in all the solvent systems used. Further storage leads to a very rapid diminution in the quantity of this unknown substance. This can be seen in the samples analysed on the 23rd and 29th days of storage. A corresponding increase in iodide radioactivity is then observed in Fig. 1. In the original autoradiogram (but unfortunately not seen as clearly in its photographic reproduction), the spot of Compound "1" was often accompanied by a very faintly radioactive zone labelled as Compound "2" (photographic reproduction does not reflect the same proportionality of radio-iodine in different spots seen in the original autoradiogram, nor is it possible to visualize spots of feeble intensity). Less than 5% of total radioactivity was counted in this fraction at any time and both Compounds "1" and "2" were observed even on the 6th day of storage of radiothyroxine. But the feature to be emphasized is the rather sudden conversion of a large fraction of thyroxine to Compound "1" in the third week of storage. A similar phenomenon was observed in

References p. 437

the storage of ^{131}I-labelled triiodothyronine between the 20th and 25th days of storage. The degradation product in this case was Compound "2" which was chromatographically identical with the less abundant product of thyroxine while Compound "1" was absent. No such breakdown, except for a slight deiodination, was observed in samples of low specific activity of both hormones when analysed after storage up to 40 days. A similar stability was observed in the aliquots of radiothyroxine and radiotriiodothyronine that were stored in the absence of any solvent.

Qualitatively identical results were obtained in all 10 samples of labelled thyroxine and 3 samples of triiodothyronine that were analysed during a period of 40 days. But the duration of storage preceding the sudden appearance in large amounts of Compounds "1" and "2" varied from one sample to another, as also the relative amounts of the decomposition products formed. In general, the effect of self-radiation became visible between the 18th and 27th days for all the different samples, while the maximum amount of Compounds "1" and "2" formed represented about 30–35% of the total radioactivity at any one time. The period of stability of the labelled substances was found to be shorter in samples of higher specific activity. A similar effect was produced by increasing the ambient temperature. Thus, in samples of thyroxine (26 mC/μmole) and triiodothyronine (32.5 mC/μmole) stored at 20° $\pm$ 3°, 22.8% and 36.3% of total ^{131}I was present as Compounds "1" and "2" respectively, by the 8th day of storage. Some variation in results could be accounted for by the different rates of change in geometry of stored solution. In Fig. 3 is presented an example of the way in which radioactivity was distributed as a function of time between thyroxine, iodide and Compound "1" in a sample of thyroxine stored at -2°C.

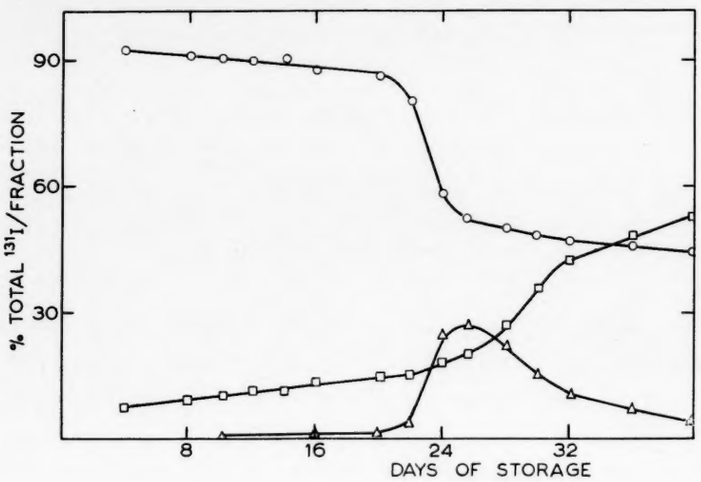


Fig. 3. Evolution of radioactivity distribution in the major radio-iodinated constituents present in a sample of ^{131}I-labelled L-thyroxine during storage for 40 days. Curves obtained from quantitative chromatographic and electrophoretic analyses. Specific radioactivity = 25.2 mC/μmole of thyroxine. o——o thyroxine; □——□ iodide; △——△ unknown compounds "1" and "2".

The slow rate of decrease in thyroxine radioactivity during the first twenty days of storage corresponded to an increase in the ^{131}I fraction as iodide. For the particular sample illustrated in Fig. 3, the 22nd, 23rd and 24th days of storage brought about a sudden and large drop in thyroxine ^{131}I fraction. This drop was not matched by an increase in the formation of inorganic iodide but in an equally sudden and quantitative appearance of the unknown Compound "1" (Compound "2" in the case of self-radiation of triiodothyronine). By this time 1 mC of ^{131}I had delivered about 60,000 rads equivalent of ionising radiation. The radiation products were short-lived and they disappeared in almost an exponential fashion, accompanied this time by a rapid

References p. 437

rise in the iodide radioactivity. In no case did the conversion of thyroxine or triiodothyronine to their radiation products go to completion at $-2°$.

Effect of external radiation

That radiation was the primary cause of breakdown of stored radioactive thyroxine and triiodothyronine was further established when the process was simulated with the action of external irradiation on the iodothyronines. The dose of external radiation employed in the first experiments was 45,000–60,000 rads because this figure was calculated to be the amount of self-radiation absorbed in samples of high-specific-activity thyroxine and triiodothyronine when the "explosive" changes occurred.

Thus when high energy X-rays from the Linear Accelerator irradiated thyroxine (Fig. 2A), about 60–90% of this substance was found to be converted to a product resembling Compound "1" obtained from self-irradiated samples at the same solute concentration. A similar breakdown of triiodothyronine (Fig. 2B) resulted in the formation of large amounts of the unknown Compound "2". That the products of external and self-radiation are identical was inferred from the failure of all chromatographic and electrophoretic methods to resolve a mixture of chromatographically isolated Compounds "1" or "2" obtained from the two types of radiation, into more than one radioactive component. The only obvious difference between self and external radiations is in the extent of degradation brought about. Radiochemical change was always more intense with external than with self-radiation when the amount of radiation delivered per mole of solute was the same (compare Figs. 1 and 2). The difference is obviously due to a higher and a constant mean dose of the external radiations employed in our experiments as compared with that derived from the β-radiation of radio-iodine (the emission of X-rays from the 4 MeV Linear Accelerator is intermittent, consisting of 2-μsec pulses occurring at a repetition frequency of approximately 350/sec. As a result the mean dose rate during each pulse is about 1400 times as high as the average of 1500 rads/min, *i.e.* 2.1 megarads/min).

The failure to observe any marked radiochemical change in samples of high-specific-activity thyroxine and triiodothyronine, when these compounds were stored in the dry state, implied that the effect of self-radiation on the compounds in solution was indirect or secondary. This implication was corroborated by results of experiments in which the iodothyronine concentration and the external radiation dose were varied as well as from the inhibition of decomposition in the presence of substances such as glycine, cysteine and serum albumin (Tables I and II).

Thus when ^{60}cobalt was employed as the source of radiation, it was observed that at any one radiation dose level the proportion of ^{131}I in the thyroxine or triiodothyronine fell inversely with respect to increasing concentrations of iodothyronine (Table I). When this is expressed as the number of molecules of either substance actually broken down, a fairly constant figure is obtained for both thyroxine and triiodothyronine. Such a relationship between concentration of irradiated substance and its degradation is characteristic of secondary or indirect effects of radiation on substances in aqueous solution[7,8]. The inhibitory effect observed on the addition of cysteine, glycine and human serum albumin (Table II) is also in agreement with an indirect reaction. Each of these substances is itself susceptible to radiochemical reactions under similar conditions and since their molar concentration was several hundred times that

References p. 437

TABLE I

RELATIONSHIP BETWEEN CONCENTRATION OF ^{131}I-LABELLED IODOTHYRONINE* AND ITS BREAKDOWN CAUSED BY γ-RADIATION FROM ^{60}COBALT (30,000 RADS)

Iodothyronine	*Concn.* $M \cdot 10^{-5}$	*Amount decomposed*	
		as % of ^{131}I	*in μmoles/l*
L-Thyroxine	1.2	58.5	7.0
	3.0	34.9	10.4
	9.0	10.0	9.0
	30.0	2.8	8.5
3,5,3′-Triiodo-L-thyronine	1.4	66.1	9.2
	3.5	42.5	14.9
	10.5	15.0	15.7
	35.0	3.5	12.2

* Specific radioactivity of thyroxine and triiodothyronine = 0.005 mC/μmole.

TABLE II

INHIBITION OF ^{60}Co-INDUCED RADIOCHEMICAL BREAKDOWN OF RADIOACTIVE L-THYROXINE

(0.005 mC ^{131}I/μmole, $3.0 \cdot 10^{-5}M$)

Radiation dose (rads)	*Inhibitor*	*Concn. mg/ml*	*% Thyroxine decomposed*
15,000	–	–	28.0
	Cysteine	0.8	3.5
	Glycine	0.7	0.0
	H.S.A.	2.0	5.1
30,000	–	–	40.5
	Cysteine	0.8	6.2
	Glycine	0.7	5.0
	H.S.A.	2.0	2.0

H.S.A. = Human Serum Albumin (Lister Institute for Preventive Medicine).
All solutions in 50% propylene glycol.
Volume of solution irradiated = 0.5 ml.

TABLE III

CHROMATOGRAPHIC MOBILITIES OF COMPOUNDS "1" AND "2" AND SUBSTANCES RELATED TO THYROXINE AND TRIIODOTHYRONINE

Substance	*Chromatographic mobility* (R_F)		
	I	*II*	*III*
Compound "1"	0.79	0.62	0.59
Compound "2"	0.81	0.74	0.66
Thyroxine	0.78	0.50	0.42
3,5,3′,5′-Tetraiodothyropyruvic acid	0.85	0.67	0.63
3,5,3′,5′-Tetraiodothyrolactic acid	0.80	0.61	0.57
3,5,3′,5′-Tetraiodothyropropionic acid	0.87	0.69	0.60
3,5,3′,5′-Tetraiodothyroacetic acid	0.87	0.68	0.60
3,5,3′,5′-Tetraiodothyroformic acid	0.88	0.68	0.61
3,5,3′-Triiodothyronine	0.80	0.69	0.50
3,5,3′-Triiodothyropyruvic acid	0.83	0.80	0.74
3,5,3′-Triiodothyropropionic acid	0.88	0.80	0.76
3,5,3′-Triiodothyroacetic acid	0.89	0.80	0.74
3,5,3′-Triiodothyroformic acid	0.88	0.79	0.74

Solvents: I = *n*-butanol–acetic acid; II = *n*-butanol–dioxane–ammonia; III = collidine–water–ammonia.

of thyroxine or triiodothyronine, their protective action is due to a "mopping up" of chemically reactive products formed by the action of ionising radiation on water.

Nature of radiochemical degradation products

A preliminary comparison of chromatographic mobilities of the two main radio-iodinated products of degradation, Compounds "1" and "2", with those of compounds structurally related to thyroxine and triiodothyronine permitted us to eliminate some of the possible answers. Since it was felt that the amino-acid grouping of either iodothyronine was more likely to be modified by the effects of ionising radiation than any other, the mobilities here illustrated are those of the deaminated analogues of the two hormones.

The R_F values given in Table III should not be taken as absolute values but are valid only for comparison because they were all determined under identical conditions and simultaneously. At first sight it appears that the chromatographic properties of Compounds "1" and "2" are not very different from the group of deaminated analogues of thyroxine and triiodothyronine, namely the propionic, acetic and formic acids. On further examination it will be seen that the R_F value of Compound "1", in the alkaline butanol and collidine systems, resembles more closely that of 3,5,3',5'-tetraidothyrolactic acid than of any other deaminated analogue of thyroxine. Electrophoretic separation on paper brought out this resemblance even more closely (Table IV).

TABLE IV

ELECTROPHORETIC MOBILITIES OF COMPOUNDS "1" AND "2" AND OF SOME ANALOGUES OF THYROXINE AND TRIIODOTHYRONINE

Substance	*Mobility (mm/100 V/18 h)*
Compound "1"	74.5
Compound "2"	35.5
Thyroxine	7.0
3,5,3'-Triiodothyronine	4.0
3,5,3',5'-Tetraiodothyropropionic acid	52.0
3,5,3',5'-Tetraiodothyrolactic acid	72.0
3,5,3',5'-Tetraiodothyroacetic acid	51.5
3,5,3'-Triiodothyropropionic acid	27.0
3,5,3'-Triiodothyroacetic acid	27.0

Electrophoresis medium: 0.075 *M* TRIS buffer, pH = 9.0.

The radioactive product of decomposition, as seen by electrophoretic mobility measurements, corresponded only to the lactic acid analogue of thyroxine.

Thus the above analytical methods suggest strongly that the main radiochemical reaction in self- and external radiation of the two iodo-amino acids is that of an oxidative deamination. Such a reaction was confirmed from the results of paper spot-test analysis summarized in Table V. For the purposes of spot-test reactions, Compounds "1" and "2" were isolated from externally irradiated samples and before any appreciable amount of radio-iodide had accumulated in the samples.

The following interpretations regarding the structure of Compounds "1" and "2"

References p. 437

were drawn from the organic group tests: (1) The negative ninhydrin and positive diazotized sulphanilic acid reactions showed the disappearance of the amino acid group and the retention of the free phenolic group of the parent iodothyronines. (2) The identical reactions of thyroxine and Compound "1" and of triiodothyronine and Compound "2" with α-nitroso-β-naphthol indicated that the effect of radiation had not been to alter in any way the distribution of iodine atoms of the two hormones (assuming that the two iodine atoms in the unlabelled 3,5 positions would be even more stable than the iodine atoms of the phenolic benzene ring). (3) Failure to react with *o*-phenylenediamine and semicarbazide indicated that the iodothyronine amino acid grouping was not replaced by an α-keto acid group. (4) The positive diazotized *p*-nitraniline reaction again revealed the presence of a free phenolic group in Compounds "1" and "2".

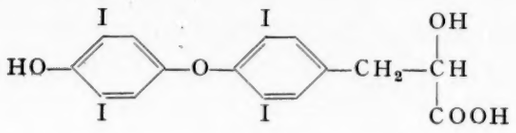
TABLE V

SPOT-TEST REACTIONS ON COMPOUNDS "1" AND "2" AND SUBSTANCES RELATED TO THYROXINE AND TRIIODOTHYRONINE

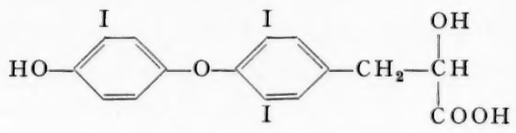
Substance	*Reaction*					
	I	*II*	*III*	*IV*	*V*	*VI*
Compound "1"	−	+	−	−	−	+ (Orange)
Compound "2"	−	+	½ +	−	−	+ (Orange)
Thyroxine	+	+	−	−	−	½ + (Pink)
3,5,3′-Triiodothyronine	+	+	½ +	−	−	½ + (Pink)
3,5-Diiodothyronine	+	+	+	−	−	½ + (Pink)
3,5,3′,5′-Tetraiodothyropyruvic acid	−	+	−	+	+	+ (Pink)
3,5,3′-Triiodothyropyruvic acid	−	+	½ +	+	+	+ (Pink)
3,5,3′,5′-Tetraiodothyrolactic acid	−	+	−	−	−	+ (Orange)
3,5,3′,5′-Tetraiodothyropropionic acid	−	+	−	−	−	+ (Red)
3,5,3′-Triiodothyropropionic acid	−	+	½ +	−	−	+ (Red)
3,5-Diiodothyropropionic acid	−	+	+	−	−	+ (Red)
3,5,3′,5′-Tetraiodothyroacetic acid	−	+	−	−	−	+ (Red)
3,5,3′-Triiodothyroacetic acid	−	+	½ +	−	−	+ (Red)
3,5-Diiodothyroacetic acid	−	+	+	−	−	+ (Red)

I = Nihydrin; II = Diazotized sulphanilic acid; III = α-Nitroso-β-naphthol; IV = Semicarbazide; V = *o*-phenylenediamine; VI = Diazotized *p*-nitraniline.

At the same time the orange colour of the complex formed was highly suggestive of the presence of a lactic acid grouping in the side-chain and an absence of any further hydroxylation of the phenolic benzene ring[5]. Combining these interpretations of spot-test results with those of the chromatographic and electrophoretic properties (Tables III and IV) of Compound "1", it can only be concluded that the major product of an indirect radiochemical action on thyroxine is the corresponding iodothyrolactic acid; although the corresponding derivative of triiodothyronine was not available, the formation of its lactic acid derivative is suggested by the results of spot tests:



3,5,3′,5′-Tetraiodothyrolactic acid
(Compound "1")



3,5,3′-Triiodothyrolactic acid
(Compound "2")

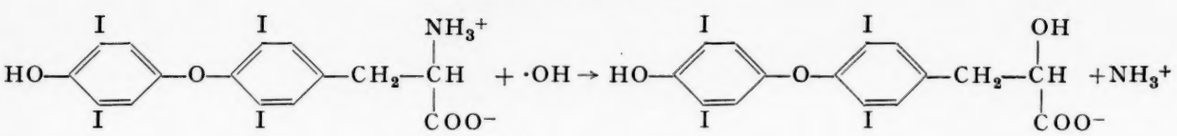
References p. 437

DISCUSSION

From the above experiments it is clear that the thyroid hormones, L-thyroxine and 3,5,3′-triiodo-L-thyronine, in dilute aqueous solutions, are susceptible to oxidation under the influence of ionising radiation. Self-radiation from β-rays of ^{131}I causes the breakdown of labelled hormones of high specific activity when stored for several days. This supposition is supported by the identity of chemical changes following external irradiation with X- and γ-rays and the stability of low-specific-radioactivity iodothyronines under similar conditions. The conditions of storage and composition of solvent employed are identical with those that many investigators would encounter on storage of ^{131}I-labelled thyroxine and triiodothyronine of high specific activity obtainable commercially.

For the following reasons it is concluded that the chemical changes produced, both by self-radiation at low rates and by external radiation at higher dose rates with X- and γ-rays, are caused by indirect or secondary effects of interaction with the solvent: (1) absence of decomposition in dried samples; (2) the low concentration of solutes in experiments on self-radiation and the inverse proportion between the chemically altered fraction of iodothyronine and its concentration (Table I); and (3) the inhibition of decomposition in the presence of relatively larger amounts of substances that are known to undergo radiochemical change under similar conditions (Table II). Thus the two iodo-amino acids react possibly with the H·, ·OH and $\cdot O_2H$ radicals or H_2O_2 produced by the action of ionising radiation on water[9] (the exact role played by proplyene glycol, in the 50% aqueous mixture used as solvent, in the nature and rate of production of free radicals or H_2O_2 is not known). There was little aeration of samples and added H_2O_2 failed to produce or enhance the reaction, which means that the $\cdot O_2H$ radical or H_2O_2 are unlikely to be involved. On the other hand, the identification of the decomposition products "1" and "2" as the corresponding lactic acid analogues (Tables III, IV and V) implies that hydroxyl radicals play an important role. The radiochemical deamination of thyroxine or triiodothyronine in the presence of radiation-induced ·OH radicals can be expressed as follows:

$$H_2O \rightarrow H_2O^+ + e$$
$$H_2O^+ \rightarrow \cdot OH + H^+$$



$$NH_3^+ + H_2O \rightarrow NH_4^+ + \cdot OH$$

Although this is the first time such a reaction has been described for iodothyronines, conversion of other α-amino acids to the corresponding lactic acids in the presence of ·OH radicals has been commonly encountered[7, 10].

It is not known exactly why in the experiments on self-radiation in samples of thyroxine and triiodothyronine of high specific radioactivity, the decomposition should begin so abruptly after a long period of relative stability (Figs. 1 and 3). Nor is it clear why the reaction rate falls just as abruptly and does not proceed to completion. This is in contrast to the almost total deamination obtained with X-rays from the Linear Accelerator (Fig. 2). The large variation in dose rates in the two cases is cer-

References p. 437

tainly a major cause of this difference and the low temperature for self-radiation is also a significant factor (all experiments with external irradiation were carried out at room temperature; experiments on storage of high-specific-activity substances at higher temperatures led to a shorter period of initial stability). An examination of Fig. 3 shows that the triiodo- and tetraiodothyrolactic acids are the precursors of the large amount of labelled iodide released, although the two iodothyronines also undergo a slow, spontaneous and direct deiodination throughout the whole period of storage. Experiments on the stability of labelled lactic acid analogues of low specific radioactivity, under identical conditions of storage, suggest that their deiodination results more from the influence of radiation than from any spontaneous instability in dilute solutions.

The instability of labelled thyroxine and triiodothyronine in solution observed by other investigators[1, 2] was very likely a manifestation of radiation breakdown, although no studies have been so far published proving the cause of the instability. The large number of radio-iodinated products obtained by LISSITZKY[1] after storage of labelled thyroxine were not all found in our chromatograms. But the formation of the acetic acid analogue claimed by him to be formed may very well be the lactic acid derivative. The work of YALOW AND BERSON[11, 12] on the radiochemical decomposition of ^{131}I-labelled human serum albumin and insulin is of interest. These authors have shown that the alterations in biological properties of these labelled proteins (such as turnover and excretion rates) after storage or after X-irradiation could be quantitatively explained on the basis of the radiochemical decomposition and an ultimate deiodination.

As regards practical aspects, the close chromatographic similarity between the lactic acid and other deaminated analogues of thyroxine and triiodothyronine is of importance. The biological conversion of labelled thyroid hormones of high specific activity to their acetic acid analogues, deduced solely on the basis of chromatographic analysis, has been the subject of a number of recent investigations. The danger of interpreting a self-radiation induced reaction as a biological transformation is hence quite obvious. This study shows that it is possible to prevent the effect of self-radiation on labelled thyroxine and triiodothyronine by storage in the dried or frozen state, and by the addition of cysteine, glycine or serum albumin at concentrations that would not be objectionable in biological investigations.

ACKNOWLEDGEMENTS

I am indebted to Dr. R. PITT-RIVERS, F.R.S., for help and advice during the course of the work. I am very grateful for generous help and advice in respect of the effects of external irradiation to Mr. F. S. STEWART, Physicist, Mount Vernon Hospital and The Radium Institute, Northwood, Middlesex. I am also indebted to Professor B. W. WINDEYER, Director of the Radiotherapy Department, Mount Vernon Hospital, for the use of the 4 MeV Linear Accelerator and ^{60}Co therapy unit. The sample of 3,5,3′,5′-tetraiodothyrolactic acid was a gift from Dr. KENNETH FLETCHER, M. R. C. Radiopathology Unit, Hammersmith Hospital, London, W. 12. All samples of high specific radioactivity were kindly provided by Dr. W. L. MONEY, Sloan-Kettering Institute for Cancer Research, New York, 21, N.Y.

References p. 437

SUMMARY

1. Chemical changes produced in dilute solutions of ^{131}I-labelled L-thyroxine and 3,5,3′-triiodo-L-thyronine by the action of self- and external radiations (high energy X- and γ-radiation) have been studied quantitatively.

2. The same major products of radiochemical decomposition, Compounds "1" and "2", were obtained from self-radiation in labelled thyroxine and triiodothyronine of high specific activity and from externally irradiated samples of low specific activity. By a combination of chromatographic, electrophoretic and spot-test analyses, Compound "1" has been tentatively identified as 3,5,3′,5′-tetraiodothyrolactic acid, with a strong suggestion for the formation of a similar derivative of triiodothyronine.

3. The kinetics of radiochemical change induced by self-radiation of the two iodothyronines have been studied over a period of storage of 40 days. Once formed, the lactic acid analogues of the hormones are rapidly deiodinated.

4. The radiochemical reaction is inhibited by storage in the dried or frozen state or by the addition of cysteine, glycine and human serum albumin.

5. The possible mechanism of the radiation-induced changes and their implications in biological work are discussed.

REFERENCES

1 S. LISSITZKY, in *Regulation and Mode of Action of Thyroid Hormones, Ciba Foundation Colloquia in Endocrinology*, 10 (1957) 181.
2 J. H. WILKINSON, W. E. SPROTT, C. H. BOWDEN AND N. F. MACLAGAN, *Biochem. J.*, 56 (1954) 215.
3 G. I. GLEASON, *J. Biol. Chem.*, 213 (1955) 837.
4 J. R. TATA, J. E. RALL AND R. W. RAWSON, *Endocrinology*, 60 (1957) 83.
5 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Electrophoresis*, Academic Press Inc., New York, 1955.
6 J. ROCHE, R. MICHEL AND J. TATA, *Biochim. Biophys. Acta*, 15 (1954) 500.
7 Z. M. BACQ AND P. ALEXANDER, *Fundamentals of Radiobiology*, Butterworths Scientific Publications, London, 1955.
8 M. BURTON, in *Elementary Chemical Processes in Radiobiological Reactions, Symposium on Radiobiology*, Wiley & Sons, New York, 1952, p. 177.
9 D. E. LEA, *Brit. J. Radiol.*, Suppl. No. 1 (1947) 59.
10 W. M. DALE, J. V. DAVIES AND C. W. GILBERT, *Biochem. J.*, 45 (1949) 93.
11 R. S. YALOW AND S. A. BERSON, *Radiology*, 66 (1956) 106.
12 R. S. YALOW AND S. A. BERSON, *J. Clin. Invest.*, 36 (1957) 44.

Received August 1st, 1958

IDENTIFICATION ET DOSAGE DE L'ACIDE 5-HYDROXYINDOLACÉTIQUE

H. WACHSMUTH ET L. VAN KOECKHOVEN

Laboratoire de Chimie et de Biochimie, Hôpital Stuivenberg, Anvers (Belgique)

L'acide 5-hydroxyindolacétique (AHIA) paraît être un constituant normal de l'urine humaine. Cependant son taux ne comporte que quelques mg par litre. UDENFRIEND[1] admet qu'il peut osciller entre 2 et 8 mg par jour. Cependant, dans certains cas pathologiques, spécialement chez les patients à tumeurs "argentaffines", ce taux pourrait atteindre 350 mg.

Rappelons que l'AHIA, qui paraît dériver de la 5-hydroxytryptamine (sérotonine), est en outre apparenté à la bufoténine (N,N-diméthyl-5-hydroxytryptamine) identifiée dans le venin de crapaud, les grains de *Piptadenia* et certains champignons vénéneux tels que les amanites. Il semble bien que le tryptophane soit à l'origine de ces dérivés indoliques. Dans un premier stade il y a transformation en hydroxytryptophane, auquel est apparenté la violacéine; ensuite en hydroxytryptamine et enfin en AHIA qu'on retrouve également dans les grains de *Piptadenia*[2]. UDENFRIEND estime que dans certains cas on trouve dans l'urine une autre substance 5-hydroxyindolique, en quantité infime.

Dans le cas qui nous occupe, les taux de l'AHIA ont varié, pour une trentaine d'échantillons s'échelonnant sur une période de 6 mois, entre 60 et 400 mg/l (la quantité d'urine excrétée journellement comportant 800 à 1800 ml). Nous avons fréquemment chromatographié les urines (et leurs liquides d'extraction). D'autre part, 3 échantillons de sang et 3 de matières fécales ont été soumis à la chromatographie.

Au cours des différentes opérations, nous avons tenté de révéler les spots à l'aide de divers réactifs. Nous nous sommes aperçus que certains de nos réactifs étaient infiniment plus sensibles que l'α-nitroso-β-naphtol et la *p*-diméthylaminobenzaldéhyde. Nous résumons brièvement ci-après nos divers essais. Certaines réactions ont été appliquées à la chromatographie et d'autres utilisées pour des déterminations quantitatives (voyez Tableau I).

EXTRACTION, ADSORPTION OU CONCENTRATION DE LA SUBSTANCE

L'extraction a été effectuée selon le mode opératoire, d'UDENFRIEND[1]. A 6 ml d'urine on ajoute 6 ml de 2,4-dinitrophénylhydrazine* à 0.5% dans HCl 2 *N*. Après 30 min on ajoute 25 ml de $CHCl_3$, on agite et centrifuge. L'extraction est répétée une deuxième fois. La solution aqueuse (10 ml) est alors extraite par 25 ml d'éther, après addition de 4 g NaCl. Agiter pendant 5 min. La solution éthérée est traitée par 1.5 ml d'une solution tampon (pH 7, phosphates), agiter pendant 5 min. 1 ml de la solution (ou un multiple) est alors traité par le réactif. Nous proposons 4 modes de dosage.

* Nous avons constaté que pour nos dosages l'emploi de la 2,4-dinitrophénylhydrazine pouvait être évité. Nous avons utilisé simplement de l'HCl 2 *N*. D'autre part, dans certains cas, nous avons effectué des extractions sur des quantités plus importantes d'urine et dans les cas pathologiques sur des quantités réduites.

Bibliographie p. 443

TABLEAU I

RÉACTIONS

Réactions effectuées sur la substance dissoute dans quelques gouttes d'alcool

Réactifs	*Indol*	*Scatol*	*Tryptophane*	*Sérotonine*	*AHIA*
p-Diméthylamino-benzaldéhyde + HCl conc. ½	rouge	brun vert	violet	lég. violet	bleu à chaud
Même réactif + $NaNO_2$	rouge	bleu	violet	violet	bleu foncé
Formol dilué + HCl conc. ½	mauve sol. dans l'alc. amylique	brunâtre	brun	brun	jaunâtre
Vanilline + H_2SO_4 conc. ½	rouge brun	violet	lég. violet	violet	violet intense
HCl conc. ½ + quelques gouttes sol. alc. furfurol	solution brunit; préc. noir après quelque temps, accéléré par élév. temp.	solution brun rouge, préc. comme l'indol	brunit, peu de préc. à froid	brunit, préc.	brunit, abondant préc. par élév. de temp.*
HCl conc. + furfurol	jaunit (à chaud préc.)	devient rouge (à chaud préc.)	devient rouge (à chaud préc.)	pas de réaction, après 10 min lég. violet et préc.	rougit et après 10 min préc.
Sol. alc. de dialdéhyde phtalique + HCl à 50%	gris vert, fluorescence bleuâtre	brun, fluorescence brune	peu coloré fluorescence brune	jaune avec fluorescence brune	color. rouge fugace (qui diffère suivant l'acide employé). Par alcali, NH_4OH ou Na_2CO_3, color. jaune, forte fluorescence (sensib. 1 : 20,000,000); avec NaOH col. verte, fluoresc. verte
Ferricyanure ferrique chlorhydrique	précipité vert bleu	précipité vert bleu	négatif	coloration verte	coloration verte + précipité
1,4-Naphtoquinone (alc.) + HCl conc.	coloration violacée + fluorescence bleue	coloration jaune + fluorescence jaune	coloration jaune + fluorescence jaune	coloration jaune + fluorescence jaune	coloration jaune intense + fluorescence jaune
Xanthydrol acétique + quelques gouttes HCl ou H_3PO_4	violet intense	lég. violet, mauve intense à chaud	mauve à chaud	lég. violet, bleu intense à chaud	violet bleu intense à chaud

* Le produit de couleur noire a été isolé. Il contient 6.2% d'azote. Vraisemblablement il y a condensation de deux molécules de AHIA avec 1 molécule, de furfurol.

Nous passerons successivement en revue les dosages à l'aide du ferricyanure ferrique, de la vanilline, de la 1,4-naphtoquinone et du xanthydrol.

L'adsorption directe de l'AHIA à partir de l'urine est pratiquement nulle lorsqu'on utilise la terre de Berckefield ou l'Al_2O_3 Brockmann. Par contre la substance se laisse adsorber par le charbon activé, mais l'élution par l'alcool ne restitue qu'environ 70% de la substance. Sur Amberlite (faible), l'AHIA est adsorbé quantitativement, mais est pratiquement irrécupérable par élution ultérieure avec NaOH 10%. Toutefois il est possible de précipiter l'AHIA de sa solution éthérée par addition d'éther de pétrole. Cependant le produit recueilli ne contient que $\pm$ 40% de la substance. On peut également alcaliniser légèrement l'urine, évaporer à sec, acidifier légèrement et l'extraire alors par l'éther. Cette technique permet d'éviter l'"outsalting". Après passage dans le tampon et réextraction par l'éther, on obtient finalement un produit dont la pureté atteint également 40%.

CHROMATOGRAPHIE

Nous nous sommes servi principalement de 3 solvants chromatographiques.

Le mélange propanol–eau (5 : 1) a été utilisé par UDENFRIEND[1]. Nous avons également trouvé un R_F de 0.74 (UDENFRIEND: 0.75). Le même solvant nous a donné un R_F de 0.38, par mouvement ascendant. La collidine–eau (125 : 44) en atmosphère saturée de NH_3 donne un R_F de 0.53 (desc.), le butanol–acétique (butanol 7, acide acétique 2 et eau 1), un R_F de 0.70 (desc.).

Dosages

A. Le ferricyanure ferrique

Mode opératoire. On ajoute dans l'ordre à

1 ml de la substance extractive (tampon)
1 ml de $K_3Fe(CN)_6$ 2%
1 ml de $FeCl_3$ 0.6%
3 ml de HCl conc.

Mesurer à 650 mμ après 30 min. Dans nos conditions opératoires 10 μg = 1.08 D.O. (1 cm).

Notons qu'il est également possible de déterminer volumétriquement l'AHIA en faisant réagir le ferricyanure en milieu alcalin (acidifier; Zn; $Na_2S_2O_3$ 0.001 *N*). Cependant les résultats ne sont pas strictement proportionnels.

Ce réactif et le mode de dosage nécessitent quelques réserves. La réaction est beaucoup plus sensible que celle à l'α-nitroso-β-naphtol (environ 40 fois). Nous avons obtenu d'autre part des résultats quantitatifs pour des dosages effectués sur l'urine pathologique. Cependant le dosage fournit des résultats erronés avec les urines normales, les quantités de liquide extractif utilisées (jusqu'à 1 ml, représentant 0.5 à 1.5 ml d'urine) étant beaucoup plus importantes et certaines impuretés que les extractions successives n'ont pas éliminées interférant avec l'AHIA. Le réactif est excellent en chromatographie.

B. La vanilline

Ce réactif nous a fourni avec l'urine telle quelle ou l'extrait une fort belle coloration violette. Les spots sont également fort bien révélés à l'aide de ce réactif.

Bibliographie p. 443

Mode opératoire. Ajouter à

1 ml de la solution à doser (extrait éthéré, repris par l'eau)
0.5 ml de solution aqueuse de vanilline (saturée)
0.5 ml de solution aqueuse de gélatine à 2%
8 ml de solution conc. de HCl (± 40%)

Laisser développer la coloration à l'abri de la lumière. Faire la lecture après 2 h à 570 mμ. La réaction a été effectuée préalablement sur l'AHIA pur.

Prise d'essai μg	*D.O. (1 cm)*	*D.O./10 μg*
7	0.064	0.091
28	0.262	0.093
84	0.772	0.092

Dans nos conditions opératoires 10 μg de AHIA = D.O. 0.092 (pour 1 cm). La réaction est ± 2.8 fois plus intense qu'avec l'α-nitroso-β-naphtol.

C. La 1,4-naphtoquinone

Un de nous a signalé antérieurement que certaines quinones réagissent en milieu neutre ou alcalin avec certaines amines ou bases azotées. La 1,4-naphtoquinone ne réagit cependant pas avec l'AHIA. Toutefois, en présence d'un acide fort on obtient une belle coloration jaune. La 1,4-naphtoquinone devient de ce fait un réactif du noyau indolique: l'indol, le scatol, la sérotonine et le tryptophane fournissent également une réaction positive*. La réaction a lieu à froid et la coloration se produit immédiatement. Le liquide présente en lumière U.V. une belle fluorescence jaune. Seul le tryptophane réagit parmi 27 acides aminés soumis à la réaction.

Mode opératoire

1.5 ml de solution alcoolique contenant l'AHIA (résidu d'extraction éthérée *ex* tampon, dissous dans l'alcool)
0.5 ml de 1,4-naphtoquinone à 0.25% (alcool)
8.0 ml H_3PO_4 85% (agiter prudemment, bulles).

Il se développe une belle coloration jaune. En lumière U.V., on observe une forte fluorescence jaune-orange. La mesure colorimétrique est effectuée après 1 h à 450 mμ.

RÉSULTATS

Prise d'essai μg	*D.O.*	*D.O./10 μg*
9.2	0.034	0.037
18.4	0.057	0.031
36.4	0.112	0.031
54.6	0.180	0.033
72.8	0.242	0.033
92.0	0.297	0.032
109.2	0.350	0.032
128.4	0.380	0.032

A 450 mμ 10 μg = D.O. 0.032 (1 cm).

* Son isomère, la 1,2-naphtoquinone, ne réagit pas, ni la 2-méthyl-1 4-naphtoquinone. La benzoquinone et l'aminoanthraquinone donnent également une réaction négative.

Bibliographie p. 443

D. Le réactif au xanthydrol

Nous avons observé que le xanthydrol en milieu acétique développe, en présence d'un peu de HCl ou d'un autre acide fort, une belle coloration bleue avec l'AHIA. La réaction est fort sensible. Elle a été appliquée de ce fait en chromatographie et à des déterminations quantitatives.

Mode opératoire. Le produit ou extrait d'urine à doser est dissous dans l'acide acétique (environ 5 ml). On ajoute 0.5 ml de réactif au xanthydrol (100 mg de xanthydrol dans 20 ml acide acétique; centrifuger au besoin). On porte au volume de 7 ml et ajoute 0.1 ml de H_3PO_4 à 85%*. On porte au bain-marie bouillant pendant 10 min et effectue la mesure alors à 650 mμ.

RÉSULTATS

Prise d'essai μg	*D.O.*	*D.O./10* μg
12.5	0.077	0.062
25	0.155	0.062
37.5	0.230	0.0615
50	0.315	0.063
100	0.635	0.0635
150	0.970	0.064

La réaction est donc $\pm$ 2.0 fois plus sensible qu'avec l'α-nitroso-β-naphtol. La réaction effectuée sur divers extraits d'urine a donné d'excellents résultats. Cependant, pour les urines normales, il y a lieu d'opérer sur une quantité d'urine ne dépassant pas sensiblement 10 ml.

Quantité trouveé à l'α-nitroso-β-naphtol (20 ml d'urine) = 24 μg (= 1.2 mg/l); au xanthydrol (10 ml d'urine) = 9 μg (= 0.9 mg/l).

COMMENTAIRES

Les dosages effectués à l'aide de vanilline, 1,4-naphtoquinone, xanthydrol et α-nitroso-β-naphtol, ont fourni des résultats comparables. Généralement, l'α-nitroso-β-naphtol conduit à des chiffres plus faibles que la vanilline, qui donne elle-même des résultats plus bas que la 1,4-naphtoquinone. Quant au xanthydrol, il a fourni des résultats plus faibles que l'α-nitroso-β-naphtol.

En ce qui concerne les déterminations chromatographiques, il ne nous a pas été possible de déceler un dérivé hydroxyindolique autre que l'AHIA. Avec le sang, nous avons cru déceler une seule fois un spot d'AHIA (dosage 110 μg %). Les matières fécales également ont donné 2 fois un résultat positif, sans qu'il soit possible d'affirmer qu'il y ait eu ou non contamination par l'urine (dosage: 0.3–0.8 mg%). En solution collidinique (descendante), nous avons obtenu fréquemment 2 spots, mais il en a été de même avec un échantillon commercial d'AHIA, tant en solution collidinique qu'en milieu acide. Ce spot supplémentaire** est révélé par l'α-nitroso-β-naphtol, la vanilline et le xanthydrol, mais pas par la 1,4-naphtoquinone.

* Préablement, la quantité requise d'acide phosphorique peut être ajoutée à l'acide acétique.

** R_F de ce spot = 0.79 (solution collidinique descendante).

Bibliographie p. 443

RÉSUMÉ

Dans le présent travail, nous nous sommes efforcés de déterminer qualitativement et quantitativement l'acide 5-hydroxyindolacétique. Divers échantillons d'urine provenant d'individus sains et d'autre part d'un patient à tumeur "argentaffine" ont été soumis à la chromatographie. Des déterminations quantitatives ont été effectuées à l'aide de plusieurs réactifs nouveaux. Occasionnellement, sang et matières fécales ont été examinés.

SUMMARY

DETERMINATION OF 5-HYDROXYINDOLEACETIC ACID

The authors have endeavoured to determine 5-hydroxyindoleacetic acid both quantitatively and qualitatively. Samples of urine of various normal persons and of a patient with an argentaffin tumour were chromatographed and quantitative determinations performed with several new reagents. Blood and faeces were also occasionally tested.

BIBLIOGRAPHIE

1 S. UDENFRIEND, E. TITUS ET H. WEISSBACK, *J. Biol. Chem.*, 216 (1955) 499.
2 S. UDENFRIEND, E. TITUS, H. WEISSBACK ET R. PETERSON, *J. Biol. Chem.*, 219 (1956) 335.

Reçu le 20 mai 1958

EFFET DU TRYPTOPHANE, DE L'ACIDE HYDROXY-3-ANTHRANILIQUE ET DU DPN* SUR L'INHIBITION PAR LA VITAMINE K DE LA RÉACTION: ACIDE HYDROXY-3-ANTHRANILIQUE → ACIDE QUINOLINIQUE

S. AURICCHIO, E. QUAGLIARIELLO ET A. RUBINO
Institut de Chimie Biologique, Université de Naples (Italie)

INTRODUCTION

Dans de précédentes études[1, 2], nous avons démontré, par des expériences *in vivo* et *in vitro*, que les vitamines K naturelles (vitamine K_1, vitamine K_2 et phtiocol) et quelques vitamines K de synthèse (ménadione ou 2-méthyl-1,4-naphtoquinone; "Synkavit" ou diphosphate sodique de la 2-méthyl-1,4-naphtohydroquinone) bloquent chez le Rat la transformation du tryptophane en acide nicotinique, au niveau d'une des étapes intermédiaires de cette conversion, ou bien au niveau de la réaction due à l'activité hydroxy-3-anthranilico-oxydasique.

Comme explication probable du phénomène, nous avons formulé l'hypothèse que les vitamines K essayées agissaient comme antimétabolites à l'égard de l'acide hydroxy-3-anthranilique, avec accumulation consécutive des métabolites de l'interrelation tryptophane–acide nicotinique qui se trouvent en amont de la réaction bloquée (acide hydroxy-3-anthranilique et hydroxy-3-quinurénine); nous avons souligné en outre la gravité de cette lésion biochimique, en ce sens que les deux *o*-aminophénols précités, tout en étant des métabolites physiologiques, peuvent, dans des conditions déterminées, en s'accumulant dans l'organisme, devenir générateurs de néoplasies[3–6].

On peut même supposer, si l'hypothèse de l'action antimétabolique de la vitamine K à l'égard de l'acide hydroxy-3-anthranilique est exacte, qu'une charge de ce métabolite ou d'un autre métabolite précurseur peut déplacer les vitamines K en rendant de nouveau perméable la voie métabolique qui conduit du tryptophane à l'acide nicotinique.

Les présentes recherches ont été effectuées précisément dans le but d'étudier l'exactitude de cette hypothèse et de déceler les mécanismes éventuels capables de diminuer *in vivo* la toxicité des vitamines K, spécialement en ce qui concerne la lésion biochimique particulière mise en évidence par nous.

PARTIE EXPÉRIMENTALE ET RÉSULTATS

On injecte par voie intramusculaire à des rats de souche non sélectionnée, d'un poids d'environ 200 g, 10 mg de "Synkavit" (diphosphate sodique de la 2-méthyl-1,4-naphtohydroquinone) de la firme Hoffmann-La Roche: une et deux heures après l'administration de Synkavit, on pratique deux injections sous-cutanées de 5 ml chacune de solution de phosphate selon Ringer-Krebs seule ou de 5 ml de solution

* Diphosphopyridine-nucléotide.

Bibliographie p. 449

Ringer-Krebs contenant du tryptophane (60 mg pro dose) ou de l'acide hydroxy-3-anthranilique de la firme Hoffmann-La Roche (10 mg pro dose). D'autres expériences sont réalisées en injectant, après l'administration de Synkavit, 20 mg de diphosphopyridine-nucléotide (I.S.I; 10 mg pro dose), qui semble participer à l'activité hydroxy-3-anthranilico-oxydasique. A d'autres rats, au contraire, on ne pratique que les deux seules injections de tryptophane ou d'acide hydroxy-3-anthranilique ou de diphosphopyridine-nucléotide, sans administration préalable de Synkavit.

Le jour suivant on répète le traitement des animaux d'après le schéma que nous venons d'exposer.

Puis, 24 h plus tard, on sacrifie par décapitation les rats traités et les rats de contrôle, du même élevage et de mêmes poids et âge; le foie, aussitôt prélevé, est homogénisé à froid dans un appareil de Potter avec une solution de phosphate selon Ringer-Krebs (3 ml/g de tissu); puis on centrifuge et le liquide surnageant est utilisé comme source d'enzyme. Les mélanges d'incubation sont ainsi préparés: 1 ml de liquide centrifugé + 3000 μg d'acide hydroxy-3-anthranilique (volume final avec Ringer 6.34 ml); simultanément on prépare des essais à blanc, sans acide hydroxy-3-anthranilique et des essais sans tissu. Les mélanges sont incubés à 37° pendant 90 min avec agitation continue. L'incubation est bloquée avec 0.66 ml d'une solution d'acide trichloracétique à 120%. Tous les essais, après addition de 1 ml d'une solution 0.02 *M* d'acide quinolinique (Bios Lab. Inc.) ou de 0.5 ml de la même solution + 0.5 ml de H_2O sont traités avec 2 mg/ml de charbon Riedel-de Haën et finalement filtrés. Pour la détermination de l'acide quinolinique on utilise la méthode de RABINOVITZ, FINEBERG ET GREENBERG légèrement modifiée[7]. Dans le Tableau I nous rapportons les résultats des expériences exécutées.

Comme il ressort des résultats rapportés dans ce tableau, le traitement par le tryptophane ou l'acide hydroxy-3-anthranilique ou le DPN supprime, dans nos conditions expérimentales, l'inhibition par le Synkavit de l'hydroxy-3-anthranilico-oxydase.

Un autre phénomène intéressant est mis en évidence par les expériences exécutées: l'activité hydroxy-3-anthranilico-oxydasique se révèle comme inhibée par le traite-

TABLEAU I

ACIDE QUINOLINIQUE FORMÉ À PARTIR DE L'ACIDE HYDROXY-3-ANTHRANILIQUE

	Nombre d'expériences	*Moyenne* μg	*S.D.*	*S.E.*
Contrôles	6	385	10.81	4.43
Synkavit	6	231	23.00	9.42
Synkavit + tryptophane	6	385	17.14	7.02
Synkavit + acide hydroxy-3-anthranilique	6	385	17.14	7.02
Synkavit + DPN	6	395.5	15.81	6.47
Tryptophane	6	357	29.69	12.16
Acide hydroxy-3-anthranilique	6	262.5	34.45	14.11
DPN	6	392	17.14	7.02

Bibliographie p. 449

ment par l'acide hydroxy-3-anthranilique seul; le DPN seul ou le tryptophane seul, aux doses employées par nous, ne semblent pas avoir d'influence sur la capacité du foie de Rat de convertir l'acide hydroxy-3-anthranilique en acide quinolinique.

Nous avons étudié ensuite, toujours chez le Rat, la capacité éventuelle du DPN *in vitro* de supprimer l'inhibition de l'hydroxy-3-anthranilico-oxydase provoquée par le Synkavit administré par voie parentérale ou par la vitamine K_3 (ménadione) ajoutée aux mélanges d'incubation. C'est pourquoi, dans un premier groupe d'expériences, nous avons traité pendant deux jours des rats, de souche non sélectionnée, d'un poids d'environ 200 g, avec du Synkavit par voie parentérale (10 mg pro die); le troisième jour on sacrifie l'animal par décapitation et on réalise des essais enzymatiques sur l'extrait de foie comme il est indiqué précédemment; à certains essais on ajoute toutefois du DPN en concentration finale $2 \cdot 10^{-5}$ *M* (temps d'incubation toujours de 90 min et dosage de l'acide quinolinique suivant la méthode de RABINOVITZ, FINEBERG ET GREENBERG [7]).

Dans le Tableau II nous rapportons les résultats des expériences effectuées.

TABLEAU II

ACIDE QUINOLINIQUE FORMÉ À PARTIR DE L'ACIDE HYDROXY-3-ANTHRANILIQUE

Rats contrôles	*Rats traités avec Synkavit*	
	sans DPN	*avec DPN*
µg	*µg*	*µg*
378	252	231
399	210	210
378	210	231
378	252	210
399	210	210
378	252	252

De ces données il résulte clairement que le DPN *in vitro* n'a pas la capacité de supprimer, dans nos conditions expérimentales, l'inhibition par le Synkavit de l'hydroxy-3-anthranilico-oxydase.

Dans d'autres expériences exécutées sur le foie de rats non traités, on préparait les mélanges d'incubation comme d'habitude, avec addition cependant de 0.4 ml de solution dans l'alcool de vitamine K_3 (Eastman Kodak; concentration finale $1 \cdot 10^{-3}$ *M*), de DPN (concentration finale variant de $2 \cdot 10^{-5}$ à $1 \cdot 10^{-3}$ *M*) et ATP (concentration finale $1 \cdot 10^{-3}$ *M*); on exécutait simultanément des essais sans DPN ni ATP, d'autres sans vitamine K_3, ni DPN, ni ATP, et enfin des essais à blanc (temps d'incubation et dosage de l'acide quinolinique comme plus haut).

Ces résultats démontrent clairement que le DPN *in vitro*, dans nos conditions expérimentales, ne supprime pas l'inhibition par la vitamine K_3 de l'hydroxy-3-anthranilico-oxydase du foie de Rat.

DISCUSSION ET CONCLUSIONS

D'après les données que nous venons de rapporter, il semble évident qu'une charge de tryptophane ou d'acide hydroxy-3-anthranilique est capable de supprimer,

Bibliographie p. 449

TABLEAU III

ACIDE QUINOLINIQUE FORMÉ À PARTIR DE L'ACIDE HYDROXY-3-ANTHRANILIQUE

Sans vit. K_3 μg	*Avec vit.* K_3 μg	*Avec vit.* K_3 + *DPN* + *ATP* μg
399	294	294
441	378	399
399	273	273
483	399	399
441	294	273
420	315	336

dans nos conditions expérimentales, l'inhibition par la vitamine K de l'hydroxy-3-anthranilico-oxydase du foie de Rat. Une explication probable de ce phénomène pourrait être justement trouvée dans l'action antimétabolique de la vitamine K à l'égard de l'acide hydroxy-3-anthranilique; ce métabolite, en effet, soit administré comme tel, soit dérivé du tryptophane administré par voie parentérale, entrerait avec succès en compétition avec la vitamine K pour la possession des récepteurs spécifiques, en la déplaçant et en annulant de cette façon l'inhibition de l'hydroxy-3-anthranilico-oxydase.

Nous serions, dans ce cas, en présence d'un mécanisme limitant l'accumulation, causée par les vitamines K, de ces *o*-aminophénols qui, comme nous l'avons déjà dit, quoique métabolites normaux de l'interrelation tryptophane–acide nicotinique, pourraient devenir, dans des conditions particulières, générateurs de néoplasies (*loc. cit.* [3–6]). Nos recherches feraient apparaître, en outre, la possibilité de diminuer *in vivo*, par le déplacement des vitamines K, les effets toxiques liés à des doses trop élevées de ces vitamines; on connaît bien aujourd'hui quelques graves syndromes toxi-hémolytiques du prématuré (hyperbilirubinémie avec ictère nucléaire, anémie de GASSER à corps inclus) dus à un hyperdosage d'analogues hydrosolubles de la ménadione [8–14] et nous-mêmes avons réussi, en effet, à démontrer dans de récentes recherches [15] que le traitement avec le tryptophane ou avec l'acide hydroxy-3-anthranilique diminuait de façon nettement significative la formation des corps inclus dans les hématies des souris traitées par le Synkavit, syndrome qui a de nombreux points communs avec celui précité de GASSER.

Si notre hypothèse était exacte, elle poserait en outre un intéressant problème: celui de l'éventuelle capacité de doses répétées d'acide hydroxy-3-anthranilique de provoquer des hypovitaminoses K et par conséquent des troubles de l'hémocoagulation.

Il faut cependant considérer que nos résultats prouveraient que le DPN *in vivo* est aussi capable de supprimer l'inhibition par la vitamine K de l'hydroxy-3-anthranilico-oxydase du foie de Rat; il est donc probable que le tryptophane et l'acide hydroxy-3-anthranilique agissent aussi en tant que précurseurs des coenzymes pyridiniques [16].

L'action anti-toxique démontrée par nous pour le DPN *in vivo* pourrait se rapporter à la capacité du DPN, qui semble participer à l'activité hydroxy-3-anthranilico-oxydasique [17], de constituer des complexes enzymatiques avec les vitamines K, en soustrayant celles-ci à l'action compétitrice avec l'acide hydroxy-3-anthranilique. L'in-

Bibliographie p. 449

capacité du DPN *in vitro* à supprimer l'inhibition par la vitamine K ou par la ménadione de l'hydroxy-3-anthranilico-oxydase du foie de Rat serait au contraire probablement due à l'impossibilité du DPN, dans ces conditions expérimentales, de constituer des complexes enzymatiques avec les vitamines K. Il est intéressant de souligner que ce comportement différent du DPN *in vivo* et *in vitro* différencie nettement l'inhibition de l'hydroxy-3-anthranilico-oxydase par les vitamines K de celle mise en évidence dans le foie de rats traités avec le 2-acétaminofluorène[18] ou porteurs de myélome d'OBERLING ET GUÉRIN en phase leucémique[19]: dans ces cas, l'inhibition est en effet, supprimée complètement par l'addition *in vitro* de DPN[20, 21].

En dernier lieu nous voudrions nous arrêter sur une autre donnée surgie au cours de nos recherches: à savoir, la capacité de l'acide hydroxy-3-anthranilique d'inhiber à lui seul et à un degré important l'activité hydroxy-3-anthranilico-oxydasique du foie de Rat.*

A la suite de ce résultat, il faut observer que dans les conditions pathologiques ou se produit un blocage de l'hydroxy-3-anthranilico-oxydase[22, 23], l'accumulation consécutive d'acide hydroxy-3-anthranilique limite encore plus l'activité hydroxy-3-anthranilico-oxydasique.**

Le phénomène acquiert encore plus de relief, si l'on tient compte que, toujours dans le domaine de l'interrelation tryptophane–acide nicotinique, en amont de l'hydroxy-3-anthranilico-oxydase, agit un autre enzyme, la tryptophano-peroxydase, dont on a démontré la nature adaptatrice; en effet, une charge de tryptophane et même de quinurénine augmenterait l'activité tryptophano-peroxydasique chez le Rat[24]. De sorte qu'est suggérée la possibilité que dans ces conditions pathologiques ou on a une conversion diminuée de l'acide hydroxy-3-anthranilique (*loc. cit.*[22, 23]), l'accumulation dans les urines des *o*-aminophénols de l'interrelation tryptophane-acide nicotinique trouve comme cause première un déficit de l'activité hydroxy-3-anthranilico-oxydasique; cette lésion serait aggravée, d'une part, par le catabolisme augmenté du tryptophane à travers les quinurénines par l'action de la tryptophano-peroxydase (exaltée par l'accumulation de l'aminoacide par utilisation réduite de l'un de ses métabolites finals, l'acide hydroxy-3-anthranilique), d'autre part, par une inhibition ultérieure de l'hydroxy-3-anthranilico-oxydase par l'accumulation de l'acide hydroxy-3-anthranilique lui-même.

RÉSUMÉ

On démontre la capacité d'une charge par voie parentérale de tryptophane ou d'acide hydroxy-3-anthranilique de supprimer l'inhibition par les vitamines K de l'hydroxy-3-anthranilico-oxydase du foie de Rat.

Le même effet est mis en évidence par des injections de DPN, tandis que le DPN *in vitro* ne supprime pas l'inhibition par les vitamines K de l'hydroxy-3-anthranilico-oxydase. Les résultats sont discutés en rapport avec l'action antimétabolique supposée de ces vitamines à l'égard de l'acide hydroxy-3-anthranilique.

---

* L'administration de tryptophane n'entraîne pas d'inhibition, peut-être du fait que l'acide hydroxy-3-anthranilique qui se forme à partir de l'aminoacide n'atteint pas un taux suffisant.

** Il est clair que ce n'est pas là le cas de l'inhibition par la vitamine K, parce que dans ces conditions c'est le rapport quantitatif entre l'acide hydroxy-3-anthranilique et la vitamine K qui règlerait l'existence de l'inhibition même.

Bibliographie p. 449

SUMMARY

EFFECT OF TRYPTOPHAN, 3-HYDROXYANTHRANILIC ACID AND DPN ON THE INHIBITION BY VITAMIN K OF THE CONVERSION OF 3-HYDROXYANTHRANILIC ACID TO QUINOLINIC ACID

It was shown that a parenteral dose of tryptophan or 3-hydroxyanthranilic acid could suppress the inhibition by K vitamins of rat liver 3-hydroxyanthranilic acid oxidase. Injection of DPN produced the same effect, whereas *in vitro* DPN does not suppress this inhibition. The results are discussed in connection with the anti-metabolic action towards 3-hydroxyanthranilic acid attributed to these vitamins.

BIBLIOGRAPHIE

[1] E. QUAGLIARIELLO, S. AURICCHIO, R. SORDINO ET M. PIAZZA, *Giorn. biochim.*, 7 (1958) 28.
[2] E. QUAGLIARIELLO, S. AURICCHIO ET B. BARONE, *Giorn. biochim.*, 7 (1958) 36.
[3] G. M. BONSER, D. B. CLAYSON ET J. W. JULL, *Brit. Emp. Cancer Compaign, Ann. Repts.*, 31 (1953) 194.
[4] E. BOYLAND ET G. WATSON, *Nature*, 177 (1956) 837.
[5] E. BOYLAND ET D. C. WILLIAMS, *Biochem. J.*, 60 (1955) v.
[6] L. MUSAJO, A. C. BENASSI ET A. PARPAJOLA, *Clin. Chim. Acta*, 1 (1956) 229.
[7] M. RABINOVITZ, R. A. FINEBERG ET D. M. GREENBERG, *Arch. Biochem. Biophys.*, 42 (1953) 197.
[8] B. LAURANCE, *Lancet*, 268 (1955) 819.
[9] T. C. MEYER ET J. ANGUS, *Arch. Disease Childhood*, 31 (1956) 212.
[10] V. M. CROSSE, T. C. MEYER ET J. W. GERRARD, *Arch. Disease Childhood*, 30 (1955) 501.
[11] J. P. BOUND ET T. P. TELFER, *Lancet*, 270 (1956) 720.
[12] K. NITSCH, *Klin. Wochschr.*, 35 (1957) 363.
[13] C. GASSER, *Helv. Paediat. Acta*, 8 (1953) 491.
[14] H. WILLI ET F. HARTMEIER, *Schweiz. med. Wochschr.*, 80 (1950) 1090.
[15] S. AURICCHIO, E. QUAGLIARIELLO ET E. MARTUCCI, *Boll. soc. ital. biol. sper.*, 34 (1958) 547.
[16] M. DUNCAN ET H. P. SARETT, *J. Biol. Chem.*, 193 (1951) 317.
[17] E. QUAGLIARIELLO, S. AURICCHIO ET E. RINALDI, *Bull. soc. chim. biol.*, Suppl. II (1957) 165.
[18] E. QUAGLIARIELLO, S. AURICCHIO ET E. RINALDI, *Boll. soc. ital. biol. sper.*, 33 (1957) 1821.
[19] E. QUAGLIARIELLO, S. AURICCHIO, A. VIOLANTE ET E. RINALDI, *Boll. soc. ital. biol. sper.*, 33 (1957) 786.
[20] E. QUAGLIARIELLO, S. AURICCHIO, A. VIOLANTE ET E. RINALDI, *Boll. soc. ital. biol. sper.*, 33 (1957) 1709.
[21] E. QUAGLIARIELLO, S. AURICCHIO, E. RINALDI ET A. RUBINO, *IVème Congr. intern. biochim. Vienne*, 1–6 septembre 1958.
[22] E. QUAGLIARIELLO ET S. AURICCHIO, *Rass. fisiopatol. clin. e terap.*, sous presse.
[23] S. AURICCHIO ET E. QUAGLIARIELLO, *Rass. fisiopatol. clin. e terap.*, sous presse.
[24] W. E. KNOX, *Brit. J. Exptl. Pathol.*, 32 (1951) 462.

Reçu le 5 septembre 1958

EFFECT OF DPN* ON THE INHIBITION OF RAT LIVER 3-HYDROXYANTHRANILIC ACID OXIDASE BY 2-ACETYLAMINOFLUORENE AND N-2-FLUORENYLDIACETAMIDE

E. QUAGLIARIELLO, S. AURICCHIO, E. RINALDI AND A. RUBINO

Institute of Biochemistry, University of Naples (Italy)

INTRODUCTION

During the investigation of tryptophan → nicotinic acid metabolism, our attention became focussed on a study by DUNNING *et al.*[1] on the relation between diet and incidence of cancer due to 2-acetylaminofluorene. They demonstrated that cancer of the bladder arose in almost all rats (Strain Fisher 344) fed with a diet containing high quantities (1.4–4.3%) of tryptophan. On the contrary, rats on a diet containing smaller amounts of tryptophan did not develop cancer of the bladder. The fact that high doses of tryptophan have to be present in the diet for the tumor to develop, reminded us of the two cancerogenic *o*-aminophenolic compounds, 3-hydroxyanthranilic acid and 3-hydroxykynurenine, which arise during the metabolic degradation of tryptophan[2]. If 2-acetylaminofluorene produces a biochemical lesion at the level of a metabolic reaction of the two compounds, a hypothesis on the mechanism of the cancerogenic action of 2-acetylaminofluorene on bladder could be established, based on the subsequent accumulation of one or both *o*-aminophenolic compounds. Our studies demonstrated in fact that the administration of 2-acetylaminofluorene produces inhibition of the reaction 3-hydroxyanthranilic acid → quinolinic acid in the rat hepatic tissue[3].

It seemed of interest to continue these studies in order to elucidate the mechanism of inhibition of 3-hydroxyanthranilic acid oxidase by 2-acetylaminofluorene. Useful information about this problem was found in the literature: experiments by WEISBERGER *et al.* in 1953[4] indicate that 2-acetylaminofluorene forms very stable complexes with liver proteins; in 1956, GUTMANN *et al.*[5] observed that it is not the 2-acetylaminofluorene that reacts with the proteins, but one of its hydroxylated derivatives. KIELLEY's studies in 1956 and 1957[6, 7] are of special interest. KIELLEY[6] has in fact demonstrated that several cancerogenic fluorene compounds inhibit glutamic dehydrogenase; N-2-fluorenyldiacetamide would therefore act by competing successfully with DPN for the enzyme[7]. On the basis of KIELLEY's investigations, it seemed probable that 2-acetylaminofluorene inhibits the reaction 3-hydroxyanthranilic acid → quinolinic acid by acting on the utilization of DPN, which seems to activate the 3-hydroxy-anthranilic acid oxidase[8]. In order to test the validity of this hypothesis, we performed new experiments with 2-acetylaminofluorene and N-2-fluorenyldiacetamide.

---

* Diphosphopyridine nucleotide.

References p. 453

EXPERIMENTAL AND RESULTS

Liver of rats of unspecified strain, weighing about 200 g, which had received 0.1–0.2% of Hoffmann-La Roche 2-acetylaminofluorene in their diet (Coward) for 2–6 days, was used. The liver was excised, immediately homogenized in the cold in a Potter blendor with Ringer-Krebs phosphate (3 ml/g of tissue), then centrifuged and the supernatant used as enzyme source.

The incubation mixtures had the following composition: 1 ml of supernatant + 3000 μg of Hoffmann-La Roche 3-hydroxyanthranilic acid + DPN (from I.S.I.) at a final concentration of $2 \cdot 10^{-5}$ *M* (made up to final volume of 6.34 ml with Ringer's solution); at the same time mixtures without DPN, without 3-hydroxyanthranilic acid and without tissue were prepared. The mixtures were incubated at 37° for 90 min with frequent shaking.

The reaction was stopped with 0.66 ml of 120% trichloracetic acid. All the samples, after addition of either 1 ml quinolinic acid, 0.02 *M* solution (Bios Lab. Inc.) or 0.5 ml of the same solution + 0.5 ml of water, were treated with 2 mg/ml of Riedel-De Haën charcoal and then filtered. Quinolinic acid was determined according to the method of RABINOVITZ, FINEBERG AND GREENBERG with slight modifications[9].

Liver of rats of the same strain, weight and age, and which had received the same diet, but without addition of 2-acetylaminofluorene, was assayed for its ability to convert 3-hydroxyanthranilic acid into quinolinic acid under identical experimental conditions.

The results of these experiments are shown in Table I.

TABLE I

FORMATION OF QUINOLINIC ACID FROM 3-HYDROXYANTHRANILIC ACID

Control rats		*Rats treated with 2-acetylaminofluorene*	
Without DPN μg	*With DPN* μg	*Without DPN* μg	*With DPN* μg
483	462	210	336
462	483	399	420
546	567	273	378
399	378	336	483
357	378	315	483
420	441	336	399
483	483	336	420

From the data of Table I it can clearly be seen that in almost all of the treated animals there was an inhibition of the hepatic 3-hydroxyanthranilic acid oxidase, which could be almost completely removed by adding DPN to the incubation mixtures; however, under identical experimental conditions, DPN never potentiates the activity of the 3-hydroxyanthranilic acid oxidase of the livers of normal rats.

In other experiments we studied the 3-hydroxyanthranilic acid oxidase activity of rats treated with parenteral injections of N-2-fluorenyldiacetamide* and the effect of DPN, added *in vitro* on this activity. Rats weighing about 200 g were treated for 4

* Our thanks are due to Prof. KIELLEY and Prof. WEISBERGER, who kindly furnished this substance.

References p. 453

days parenterally with N-2-fluorenyldiacetamide (40 mg in 2 ml sterile olive oil, per day); on the fifth day the treated animals and the controls, which had received 2 ml of olive oil per day for 4 days, were killed by decapitation and the liver 3-hydroxyanthranilic acid oxidase determined as described above.

The results of these experiments are shown in Table II.

TABLE II

FORMATION OF QUINOLINIC ACID FROM 3-HYDROXYANTHRANILIC ACID

Control rats		*Rats treated with N-2-fluorenyldiacetamide*	
Without DPN	*With DPN*	*Without DPN*	*With DPN*
μg	*μg*	*μg*	*μg*
525	504	315	504
504	504	336	504
462	483	252	441
483	483	294	441
525	525	399	525
546	525	441	441

The data reported in Table II show that the treatment with N-2-fluorenyldiacetamide inhibits the rat liver 3-hydroxyanthranilic acid oxidase activity; DPN added to the incubation mixtures brings the enzymic activity back to normal values.

DISCUSSION AND CONCLUSIONS

In a former paper[3] we showed that rat hepatic tissue shows a strong inhibition of the reaction 3-hydroxyanthranilic acid → quinolinic acid after administration of 2-acetylaminofluorene. It is of interest that the inhibition of the 3-hydroxyanthranilic acid oxidase activity can be observed already 24 h after the first administration of 2-acetylaminofluorene, whereas according to WILSON *et al.*[10], tumors appear only after 3–4 months. Therefore the biochemical lesions caused by the carcinogens would be precocious.

The mechanism by which 2-acetylaminofluorene and N-2-fluorenyldiacetamide produce a lesion at the level of the 3-hydroxyanthranilic acid oxidase activity can be explained by our present results: these carcinogenic compounds would act by inhibiting the utilization of the pyridine coenzyme. The addition of DPN brings the hepatic tissue 3-hydroxyanthranilic acid oxidase activity of rats treated with these carcinogens back to the original level.

Our studies therefore confirm KIELLEY's data[6, 7] on the inhibitory influence of fluorene carcinogens on the utilization of the pyridine coenzymes. Moreover, these investigations lead to the following important conclusion: the inhibition of the reaction 3-hydroxyanthranilic acid → quinolinic acid is observed both in hepatic tissue of rats bearing Oberling, Guérin and Guérin myeloma at the leukemic phase[11, 12] and in the hepatic tissue of rats treated with 2-acetylaminofluorene and N-2-fluorenyldiacetamide. In both cases this inhibition is removed by DPN.

SUMMARY

It is shown that *in vitro* DPN removes the inhibition of rat liver 3-hydroxyanthranilic acid oxidase produced by 2-acetylaminofluorene and N-2-fluorenyldiacetamide.

References p. 453

REFERENCES

1 W. F. DUNNING, M. R. CURTIS AND M. D. MANN, *Cancer Research*, 10 (1950) 454.
2 E. BOYLAND AND G. WATSON, *Nature*, 177 (1956) 837.
3 E. QUAGLIARIELLO, S. AURICCHIO AND E. RINALDI, *Boll. soc. ital. biol. sper.*, 33 (1957) 1821.
4 E. K. WEISBERGER, J. R. WEISBERGER AND H. P. MORRIS, *Arch. Biochem. Biophys.*, 43 (1953) 474.
5 H. R. GUTMANN, J. H. PETERS AND J. G. BURTLE, *J. Biol. Chem.*, 222 (1956) 373.
6 R. K. KIELLEY, *Biochim. Biophys. Acta*, 21 (1956) 574.
7 R. K. KIELLEY, *J. Biol. Chem.*, 227 (1957) 91.
8 E. QUAGLIARIELLO, S. AURICCHIO AND E. RINALDI, *Bull. soc. chim. biol.*, Suppl. II (1957) 165.
9 M. RABINOVITZ, R. A. FINEBERG AND D. M. GREENBERG, *Arch. Biochem. Biophys.*, 42 (1953) 197.
10 R. H. WILSON, F. DE EDS AND D. J. COX, *Cancer Research*, 1 (1941) 595.
11 E. QUAGLIARIELLO, S. AURICCHIO, E. RINALDI AND A. VIOLANTE, *Clin. Chim. Acta*, 3 (1958) 381.
12 E. QUAGLIARIELLO, S. AURICCHIO, E. RINALDI AND A. VIOLANTE, *Clin. Chim. Acta*, 3 (1958) 441.

Received september 5th, 1958

SHORT COMMUNICATIONS

The assay of arylsulphatases A and B in human urine

Two soluble arylsulphatases (arylsulphatases A and B) are known to occur in human tissues[1]. They are readily separated from a third, insoluble enzyme (arylsulphatase C), from which they are easily distinguished by virtue of different substrate specificities and different response to certain inhibitors such as phosphate ions[2]. However, although the presence of the two soluble enzymes may be demonstrated by electrophoresis or solvent fractionation techniques, it has not until now been possible to obtain a direct quantitative measure of the relative amount of each in any tissue. This has been because each enzyme exhibits some activity under the optimum experimental conditions for the other, because no selective inhibitors were known and because a marked non-linear enzyme concentration–activity relationship is exhibited by arylsulphatase A[3].

Recent studies have allowed us to overcome these difficulties and methods have been developed whereby arylsulphatases A and B may be determined independently even when present in low concentrations. Detailed enzymological considerations will be published elsewhere, but the following principles are involved in the determination of arylsulphatase A in the presence of arylsulphatase B:

(*a*) "normal" kinetics are exhibited by arylsulphatase A at low substrate concentrations in the presence of $2.5 \cdot 10^{-4}$ *M* sodium pyrophosphate[4];

(*b*) considerable inhibition of arylsulphatase B occurs under these conditions;

(*c*) arylsulphatase B is specifically inhibited by chloride ions when potassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) is the substrate, whereas arylsulphatase A is not thus inhibited. The determination of arylsulphatase B in the presence of arylsulphatase A is based on the fact that arylsulphatase A exhibits only a small residual activity after the first 20 min of the reaction when incubated at high substrate concentration in the presence of barium ions and in the absence of interfering ions. Moreover this residual activity is linear and proportional to the intercept obtained by extrapolating this line back to zero time. Barium ions do not affect the activity of arylsulphatase B.

The present communication is concerned with the determination of the soluble

References p. 455

arylsulphatases of human urine. The presence of both arylsulphatase A and arylsulphatase B in cell-free urine has already been demonstrated by paper electrophoresis[5]. Assays of arylsulphatase activities in normal and pathological urines have already been attempted[6, 7] and considerable increases in the activity in the urines of patients suffering from certain pathological conditions have been claimed. However very long incubations under arbitrary conditions in the presence of endogenous interfering ions were used in these determinations and no account was taken of the fact that more than one arylsulphatase was present. The quantitative results may therefore not be very significant.

Using the procedure described below for the determination of the endogenous arylsulphatases in human urine it has been possible also to recover quantitatively added amounts of purified human arylsulphatases A and B from urine.

Samples of urine (stored at 0° during collection), were adjusted to pH 6.0–6.3 before centrifuging to remove cellular debris. Portions (4 ml) were dialysed with stirring for 18 h against several changes of tap-water at 0–2° to remove interfering ions. The volumes of the dialysed samples were adjusted to 6 ml with tap-water and determinations of the arylsulphatases made as follows:

Arylsulphatase A was determined by incubating 0.5 ml of dialysed urine with 0.5 ml of reagent A (0.01 *M* NCS in 0.5 *M* sodium acetate–acetic acid buffer containing $5 \cdot 10^{-4}$ *M* $Na_4P_2O_7$ and 10% w/v sodium chloride, pH 5.0). After 1 h at 37.5°, 1.5 ml *N* NaOH was added and the liberated 4-nitrocatechol determined by measurement of the absorption (T) at 515 mμ with the Hilger Uvispec spectrophotometer using 1-cm cells. An appropriate control determination (C) was also made in which dialysed urine and reagent were incubated separately and only mixed immediately before the addition of *N* NaOH. The amount of 4-nitrocatechol liberated in μg/h/ml of urine is given by the following expression:

$$\frac{(T - C) \times 155 \times 10^6 \times 2.5 \times 6}{12{,}400 \times 10^3 \times 0.5 \times 4} = (T - C) \times 94$$

Where 155 and 12,400 are respectively the molecular weight and molecular extinction coefficient of 4-nitrocatechol. The other factors arise from the various dilutions of the urine as a result of the dialysis and assay procedures.

Under the above conditions the activity of the arylsulphatase B which is also present is 2% of its activity as determined under optimal conditions using the reagent B described below. However in the case of normal urine the appropriate correction is so small that it can be neglected.

Arylsulphatase B was determined by incubating 1.2 ml of dialysed urine with 1.2 ml of reagent B (0.05 *M* NCS in 0.5 *M* sodium acetate–acetic acid buffer containing 10^{-2} *M* barium acetate, pH 6.0) at 37.5°. Portions (1 ml) were withdrawn after 30 min and 90 min, added to 1.5 ml *N* NaOH and the liberated 4-nitrocatechol measured spectrophotometrically as before. Appropriate control determinations were also made. The corrected spectrophotometric readings ($E_{30\ min}$ and $E_{90\ min}$) were plotted against time and a line through these two points was extrapolated back to zero time. The intercept so obtained (X) is an approximate measure of the arylsulphatase A present and is proportional to the contribution which arylsulphatase A makes to the linear part of the reaction curve. It has been established that the value of the intercept (X)

References p. 455

TABLE I

Urine	*Volume ml*	*Arylsulphatase A 4-nitrocatechol liberated*		*Arylsulphatase B 4-nitrocatechol liberated*		*Ratio A:B*
		μg/ml/h	*mg/24h sample/h*	*μg/ml/h*	*mg/24h/sample/h*	
1	1500	11.6	17.4	1.9	2.8	6.1
2	1700	9.1	15.5	1.4	2.4	6.5
3	1500	20.3	30.5	2.3	3.5	8.8
4	1500	22.2	33.3	1.3	2.0	17.1
5	1850	7.4	13.7	1.3	2.4	5.7
6	1300	7.8	10.2	1.5	2.0	5.2
7	1400	19.6	27.4	2.8	3.9	7.0
8	750	4.5	3.4	1.1	0.8	4.1
9	1450	3.8	5.5	0.3	0.4	12.7
10	1200	8.7	10.4	0.5	0.6	17.4

divided by 5 represents the maximum contribution made by arylsulphatase A to the activity of the urine between the 30th and the 90th min under these conditions. The activity of arylsulphatase B over one hour may therefore be expressed as ($E_{90\ min} - E_{30\ min} - 0.2\ X$) and the amount of 4-nitrocatechol liberated in μg/h/ml of urine is given by the following expression which is analogous to that derived for arylsulphatase A: ($E_{90\ min} - E_{30\ min} - 0.2\ X$) × 94.

Using the above procedure, 24 h samples of urine from healthy adult males have been assayed for arylsulphatases A and B. Results obtained for some typical samples are given in Table I.

Good recoveries of added arylsulphatases A and B have also been obtained from human serum by this procedure and work is in progress to determine the amounts of these enzymes present in normal and pathological sera as well as in pathological urines.

One of us (H. B.) is grateful to the Medical Research Council for a Research Assistantship.

H. BAUM*
Department of Biochemistry, University of Wales, Cardiff (Great Britain) K. S. DODGSON
B. SPENCER

1 K. S. DODGSON, B. SPENCER AND C. H. WYNN, *Biochem. J.*, 62 (1956) 500.
2 K. S. DODGSON AND B. SPENCER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 4, Interscience Publishers, Inc., New York, 1957.
3 H. BAUM, K. S. DODGSON AND B. SPENCER, *Biochem. J.*, 69 (1958) 567.
4 H. BAUM AND K. S. DODGSON, *Biochem. J.*, 69 (1958) 573.
5 K. S. DODGSON AND B. SPENCER, *Clin. Chim. Acta*, 1 (1956) 478.
6 E. BOYLAND, D. M. WALLACE AND D. C. WILLIAMS, *Biochem. J.*, 56 (1954) xxix.
7 L. M. DZIALOSZYNSKI, *Clin. Chim. Acta*, 2 (1957) 542.

Received September 27th, 1958

* Present address: Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, London, W.C.1.

Fluorimetric method for differential estimation of the 3-O-methylated derivatives of adrenaline and noradrenaline (metanephrine and normetanephrine)

Soon after the observation of ARMSTRONG AND MCMILLAN[1], that 3-methoxy-4-hydroxymandelic acid is a major metabolic product of adrenaline and noradrenaline, AXELROD[2] demonstrated the methylation of these catechol amines at the 3-hydroxy position *in vitro* and *in vivo*. AXELROD *et al.*[3] observed that 70 % of adrenaline and noradrenaline injected into rats was converted to the corresponding 3-O-methylated derivatives, which were called metanephrine and normetanephrine. In the brains of rats treated with iproniazid, AXELROD[4] was able to detect normetanephrine in a concentration of 0.1 to 0.2 μg/g tissue. No normetanephrine could be detected in the brains of untreated rats.

For the estimation of metanephrine and normetanephrine after injection of the corresponding catechol amines, AXELROD *et al.* utilized the fluorescence of these compounds in the ultraviolet region. (For the detection of normetanephrine in brain a colour reaction on paper chromatograms was used only.) This fluorescence is relatively weak. Furthermore, the fluorescence characteristics are the same for metanephrine, normetanephrine, and a number of related substances, including adrenaline, noradrenaline, and dopamine. Therefore, the fluorescence of metanephrine and normetanephrine is of limited usefulness for estimation purposes.

As is well known, adrenaline and noradrenaline can be easily oxidized to the red indole derivatives, adrenochrome and noradrenochrome, respectively, which can then be rearranged in alkali, in the absence of oxygen, to the strongly fluorescent trihydroxyindoles, adrenolutine and noradrenolutine respectively. The fluorescence characteristics of these compounds differ enough to allow differential estimation. We have now observed that if metanephrine or normetanephrine are treated according to this principle, a strong fluorescence develops. The activation and fluorescence spectra of the fluorophores of metanephrine and normetanephrine prepared in this way, are identical with those of adrenolutine and noradrenolutine respectively; the fluorescence intensity is somewhat weaker. Metanephrine and normetanephrine do not seem to be as easily oxidized as the corresponding catechol amines. For example, potassium ferricyanide in the concentration usually employed in this laboratory for the oxidation of adrenaline and noradrenaline[5], cannot be used for the preparation of the fluorophores of metanephrine and normetanephrine. Iodine has been found to be a suitable oxidant for this purpose. The relative resistance to oxidation of the methylated derivatives is also shown by oxidation with iodine at different pH. With adrenaline and noradrenaline the fluorescence obtained after oxidation at pH 5 and 7.2 was about equally strong; with metanephrine and normetanephrine fluorescence was observed after oxidation at pH 7.2 only. Differentiation between the catechol amines and their methylated derivatives is thus possible.

When iodine is used for oxidation, dopamine is oxidized to a red indole derivative, which on rearrangement in alkali yields a compound with fluorescence characteristics

References p. 457

indistinguishable from those of the fluorophores of noradrenaline and normetanephrine. In order to eliminate the interference of dopamine, we decided to make a final pH adjustment to about 5.3 (by addition of acetic acid) after the treatment with alkali. This pH adjustment results in a drop in the wavelengths of activation and fluorescence for all the compounds mentioned. However, this drop is much more pronounced for dopamine than for the other compounds[6] (Table I).

TABLE I

WAVELENGTH PEAKS OF ACTIVATION AND FLUORESCENCE BEFORE AND AFTER FINAL pH ADJUSTMENT

Activation/fluorescence peaks in mμ (uncorrected instrumental values). Aminco-Bowman Spectrophotofluorometer.

	Alkaline	*pH 5.3*
Adrenaline	425/545	420/530
Metanephrine	425/545	420/530
Noradrenaline	410/535	395/515
Normetanephrine	410/535	395/515
Dopamine	410/535	345/410

For differentiation between metanephrine and normetanephrine the differences in fluorescence characteristics can be utilized (Table I).

Procedure. Adjust the pH of the sample to about 7.2. In a test tube add to 1 – 3 ml sample (0.1 – 1 μg metanephrine or normetanephrine): 0.5 ml 0.1 *M* phosphate buffer pH 7.2, water to give a total volume of 3.8 ml, and 0.05 ml 0.02 *N* iodine solution. After 5 min add 0.5 ml 4.5 *N* NaOH containing 0.2 % ascorbic acid (freshly prepared solution). After another 5 min add 0.6 ml 5 *N* acetic acid. Read samples immediately in an Aminco-Bowman spectrophotofluorometer at the wavelengths indicated in Table I.

For oxidation at pH 5, *M* acetate buffer, pH 5, is used instead of the phosphate buffer.

ÅKE BERTLER
ARVID CARLSSON
EVALD ROSENGREN

Department of Pharmacology, University of Lund (Sweden)

[1] M. D. ARMSTRONG AND A. MCMILLAN, *Federation Proc.*, 16 (1957) 146.
[2] J. AXELROD, *Science*, 126 (1957) 400.
[3] J. AXELROD, J. K. INSCOE, S. SENOH AND B. WITKOP, *Biochim. Biophys. Acta*, 27 (1958) 210.
[4] J. AXELROD, *Science*, 127 (1958) 754.
[5] Å. BERTLER, A. CARLSSON, AND E. ROSENGREN, *Acta Physiol. Scand.*, 44 (1958) 273.
[6] A. CARLSSON AND B. WALDECK, *Acta Physiol. Scand.*, 44 (1958) 293.

Received September 25th, 1958

Ethylene and propylene glycol in the pre-staining of lipoproteins for electrophoresis

During recent years a great deal of interest has been shown in the determination of serum lipoproteins by paper or starch electrophoresis. This was usually accomplished by a post-staining method, for example, that of SWAHN [1] in which Sudan Black B is the lipid stain used.

In order to avoid the inconveniences inherent in this technique, such as the problem of washing the excess dye from the paper, the intense background in the developed ionogram, etc., MCDONALD AND BERMES [2, 3] developed a pre-staining method in which 95% ethyl alcohol was used as a solvent for Sudan Black B. Recently, SOLINAS *et al.* [4] reported the use of diacetin as a solvent for Sudan Black B in order to avoid the difficulties, said by some investigators, to be encountered in the use of alcohol. These include evaporation of excess alcohol, and possible denaturation of the lipid–protein complex at higher alcohol concentrations.

The technique described by SOLINAS was tried in our laboratory and although good results were obtained it was found that the diacetin solution was difficult to handle, expecially because of its high viscosity. In our attempt to overcome this difficulty, solvents other than ethyl alcohol were re-investigated. After several trials it was found that both propylene and ethylene glycol possessed some inherent advantages as compared to the solvents previously used. A review of these developments has been presented recently [5]. Due to the interest shown by clinical chemists in the report, it was felt that a brief account of the modified procedure for pre-staining lipoproteins should be published.

Solutions of Sudan Black B were prepared by heating ethylene or propylene glycol to 100–110° and then adding, to 100 ml of the solvent, 1 g of the dye. The mixture was thoroughly stirred for 5 min and then filtered while still hot through Whatman No. 2 filter paper. After cooling the filtered solution to room temperature it was re-filtered through the same kind of paper. The concentration of the final solution was approximately 0.5 g% with respect to the dye. Care must be taken not to exceed 110° when preparing these solutions or else a useless gelatinous mixture will result.

For pre-staining the serum lipoproteins, the following procedure gave good results: to 0.50 ml of serum add slowly and with constant, gentle mixing 0.10 ml of the saturated dye solution. Let the mixture stand for 45 min at room temperature and then remove the excess dye by centrifugation (Int. Clinical Centrifuge Model CL) at maximum speed for 15 min. Aliquots of 5–30 μl of this pre-stained serum can be streaked on the paper strips, by means of a micropipet.

Ionographic separations were carried out employing the Precision Ionograph*, a horizontal strip type of apparatus, using Whatman 3 MM filter paper strips, 2 cm in width. 20 μl of the pre-stained serum was applied to the strips and the separations were carried out at 25°. Other conditions were: veronal buffer pH 8.6; ionic strength

* Precision Scientific Company, Chicago, Ill.

References p. 459

0.05; potential gradient 10 V/cm; time of run, 2 h. After this period of time the α-lipoprotein fraction had moved 4 cm from the origin and the β-lipoprotein fraction moved about 1.5 cm.

After the ionographic separations the strips were removed and the fractions eluted with a solution of 20%, by volume, acetic acid in 95% ethyl alcohol. The color was then read at 590 mμ in a colorimeter. It is also possible to dry the strips and then scan them. The lipoprotein zones on the ionograms appeared as blue-black areas against an almost white background (Fig. 1).

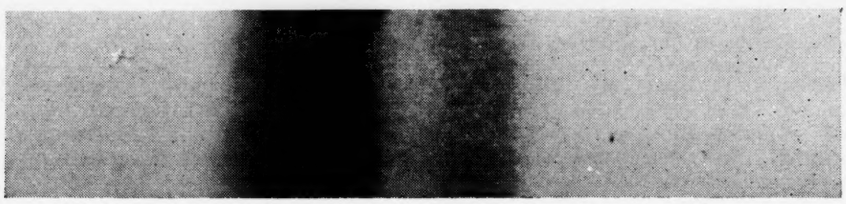


Fig. 1. Ionogram of normal serum lipoproteins. Middle (dark) zone, β-lipoprotein; lighter zone, to the right, α-lipoprotein; thin dark line, to the left of β-lipoprotein zone, point of origin; lighter zone extending from left of β-lipoprotein through the point of origin, chylomicrons.

From the patterns obtained no evident signs of denaturation were noted when the saturated dye solution was added to the serum in the ratios from 1:10 to 1:6, although best results were obtained at the ratio of 1:5.

It was also noted that the α/β ratio was practically the same in the range specified above. When different amounts of the pre-stained serum (in the ratio of 1:5) were added to the paper strips a good agreement with the Beer-Lambert law was found for both liproprotein factions, when the amount of serum used was in the range between 5 to 30 μl.

The Graduate School and Stritch School of Medicine,
Loyola University, Chicago, Ill. (U.S.A.)

H. J. McDONALD
L. P. RIBEIRO*

[1] B. SWAHN, *Scand. J. Clin. & Lab. Invest.*, 4 (1952) 98.
[2] H. J. McDONALD AND E. W. BERMES, JR., *Biochim. Biophys. Acta*, 17 (1955) 290.
[3] H. J. McDONALD, *Ciba Foundation Symposium, Paper Electrophoresis*, 1956, p. 183.
[4] P. SOLINAS, R. BETTI AND E. F. P. DI LEO, *Clin. Chim. Acta*, 2 (1957) 586.
[5] H. J. McDONALD AND E. W. BERMES, JR., *Abstr. papers presented at 10th Anniversary Meeting of Am. Assoc. Clin. Chemists, Iowa City, Iowa, September 1958*, p. 28.

Received September 30th, 1958

* Post-Graduate Research Fellow, 1957–58. Permanent address: Biochemical Laboratory, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. Acknowledgement is made of a grant for travel from the National Research Council of Brazil.

ANNOUNCEMENT

FOURTH INTERNATIONAL CONGRESS ON CLINICAL CHEMISTRY

Edinburgh, August 14th–19th, 1960

The ASSOCIATION OF CLINICAL BIOCHEMISTS (OF GREAT BRITAIN) invites you to attend the Fourth International Congress on Clinical Chemistry, to be held at Edinburgh, under the Presidency of Sir RUDOLPH A. PETERS, F.R.S., from 14th to 19th August, 1960. Four days will be devoted to scientific sessions and one to an exhibition of equipment, informal discussions, and recreational excursions. The Congress has been timed so that it is followed immediately by the Edinburgh International Festival of Music and Drama.

Congress Committee

Chairman: C. P. STEWART, D.Sc., Ph.D.
Secretary: S. C. FRAZER, M.B., Ph.D.

Members: R. GADDIE, B.Sc., Ph.D., F.R.I.C.; A. P. KENNY, B.Sc., Ph.D., A.R.I.C.; E. J. KING, M.A., Ph.D., D.Sc., F.R.I.C.; A. L. LATNER, D.Sc., M.D., M.R.C.P., F.R.I.C.; A. L. TÁRNOKY, B.Sc. (Tech.), Ph.D.; J. H. WILKINSON, B.Sc., Ph.D., F.R.I.C.

Programme

The scientific sessions will include four major symposia, with invited speakers, the provisional topics being: (1) Plasma Protein Turnover, (2) The Mechanisms of Urine Production, (3) Congenital Abnormalities of Metabolism, (4) Clinical Enzymology.

For other sessions offers of short papers or demonstrations will be invited, and it is suggested that they should bear special reference to the following subjects: (1) Recent Major Advances in Clinical Chemical Analysis. (2) Lipids and Lipoproteins. (3) Chemistry of Endocrine Abnormalities. (4) Biochemistry of the Newborn. (5) Biochemistry of the Nervous System. (6) Metabolic Aspects of Bone Disease. (7) Toxicology in Clinical Chemistry.

Papers may be presented in English, French or German, but it is suggested that English is likely to be most generally understood. There will be no simultaneous interpretation service.

A number of social events will be arranged for the evenings, and a special programme will be organized for Associate Members not attending the scientific sessions.

General information

1. *Hotel and travel agency.* Thomas Cook and Son and Wagon-Lits/Cook have been appointed official travel agents to the Congress and will offer special facilities for travel arrangements, hotel accommodation and excursions. A limited amount of hostel accommodation will be available.

2. *Detailed programme.* The detailed programme, together with application forms for membership, submission of papers, hotel reservations and excursions will be issued in the early autumn 1959.

3. *Communications.* The Congress committee will appreciate your co-operation in bringing this announcement to the notice of your colleagues. All enquiries regarding the Congress should be addressed to Dr. S. C. FRAZER, Fourth International Congress on Clinical Chemistry, Clinical Laboratory, Royal Infirmary, Edinburgh, Scotland.

CLINICA CHIMICA ACTA

Vol. 4, No. 3 May 1959

CONTENTS

CLINICA CHIMICA ACTA

SUBSCRIPTIONS should be sent to:

ELSEVIER PUBLISHING COMPANY
110-112 Spuistraat, Amsterdam-C. (The Netherlands)

Subscription price: $ 19.50 or £ 6.15.0 or Dfl. 72,—per Volume (post free), comprising 6 bimonthly issues of approx. 150 pages.

For advertising rates apply to the publishers.

ELSEVIER PUBLISHING CO., AMSTERDAM
Printed in The Netherlands by
N.V. DRUKKERIJ G. J. THIEME, NIJMEGEN